UNIVERSITY OF ALBERTA

SAMPLE TREATMENT FOR ARSENIC SPECIATION

BY

ALFONSO MONTILLA

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

DEPARTMENT OF CHEMISTRY

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled SAMPLE TREATMENT FOR ARSENIC SPECIATION submitted by ALFONSO MONTILLA in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Stability of arsenosugars towards decomposition was studied. Seaweed samples were extracted with methanol-water (1:1) and subsequently digested under different treatments. Total arsenic concentrations in non-digested and digested extracts were determined by FI-HG-AAS with or without microwave-assisted decomposition with $K_2S_2O_8$/NaOH. It was found that cooking, mild acid digestion or *in vitro* gastrointestinal digestion did not decompose arsenosugars.

Enzyme hydrolysis with polysaccharidases and sequential extraction using bath and probe sonication were explored as alternatives for improving extraction efficiencies of arsenic from seaweed samples. Extraction efficiencies from both treatments were determined by quantifying the total arsenic content in extracts, residues and samples after microwave-assisted decomposition with $K_2S_2O_8$/NaOH and FI-HG-AAS detection. None of these treatments *per se* improved the extraction efficiencies of arsenic when compared to those of water-methanol extraction. However, the sequential extraction approach (bath&probe sonication ± enzyme hydrolysis) yielded extraction efficiencies (85-90%) comparable to those reported in the literature in the same kind of samples (80-90%). Both treatments did not change the native arsenic species in the samples analyzed.

To my parents, sisters, brothers and friends

for their encouragement,

love and support

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SYMBOLS AND ABBREVIATIONS

SYMBOL	DESCRIPTION
As^{III}	arsenite
As^{V}	arsenate
MMAA	monomethylarsonic acid
DMAA	dimethylarsinic acid
TMAO	trimethylarsine oxide
TeMA	tetramethylarsonium ion
AB	arsenobetaine
AC	arsenocholine
SAM	S-adenosylmethionine
TEAH	tetraethylammonium hydorxide

FI-HG-AAS	flow injection hydride generation atomic absorption spectrometry
HFS	hydride-forming species

CHAPTER 1

INTRODUCTION

1.1 ARSENIC IN THE MARINE ENVIRONMENT

Arsenic, the 20 th most abundant element in the earth crust, is widely distributed in the environment because of its natural existence (volcanism) and mobilization through human and natural activities. Human activities involve production and use of arsenical pesticides, use of feed additives for poultry and pigs, glass making, among others. Weathering, dissolution in water and biological activities are examples of natural activities. It has been estimated that the rate of arsenic mobilization by human activities is about three times that by natural means[1, 2, 3].

The extensive use of arsenic compounds releases substantial amounts of arsenic in the environment. As a result, this element is found in a large variety of environmental and biological samples in variable amounts and in a great variety of species[2,3]. Table 1.1 shows some arsenic compounds and their range of use.

Arsenic in seawater is usually found at a concentration of 2 μg L^{-1}, with seasonal variations in the case of surface seawaters, due to biological uptake[3]. Arsenate (As^{V}) is the major arsenic compound present; arsenite (As^{III}), monomethylarsonic acid (MMAA) and dimethylarsinic acid (DMAA) are present at lower levels usually fewer than 10% of the total arsenic concentration[2].

Table 1.1: Common arsenic compounds and their uses (adapted from Reference 2)

Compound	Formula	Use
Lead arsenate	$PbHAsO_4$	Insecticide
Cupric arsenite	$CuHAsO_3$	Pigment
Arsenic oxides	As_2O and As_2O_5	Glass industry
Arsanilic acid	$C_6H_8AsNO_3$	Animal growth
Arsenic sulphide	As_4S_4	Fireworks
Gallium arsenide	GaAs	Semiconductors
Alkyldichloroarsines	$RAsCl_2$	Chemical weapons
Melarsoprol	$C_{12}H_{15}AsN_6OS_2$	Medicine (intestinal amebiasis)

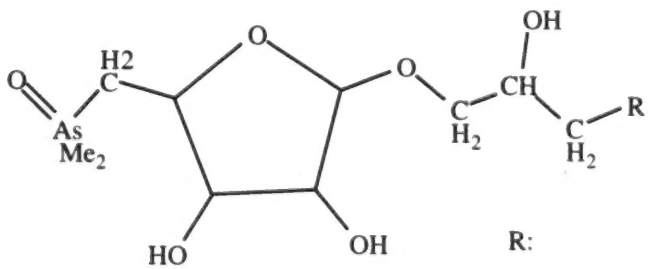
Arsenic compounds with one to four methyl groups are common constituents of marine samples[4]. Major species are MMAA, DMAA, trimethylarsine oxide (TMAO) and tetramethylarsonium ion (TeMA).

Inorganic arsenic and methylated arsenic species such as MMAA, DMAA and TMAO readily produce arsines upon reduction with sodium borohydride ($NaBH_4$) in acidic medium, a method that is commonly used for determination of arsenic[5]. Also, because of the large differences in their anionic/cationic properties, these arsenic species have been separated by chromatographic techniques[4]. Table 1.2 and Figure 1.1 show names, abbreviations and structures of some arsenic compounds of environmental and biological significance.

Table 1.2: Arsenic compounds of environmental and biological importance (adapted from Reference 2)

Name	Abbreviation	Formula
I. Arsenite	As^{III}	$As(OH)_3$
II. Arsenate	As^{V}	H_3AsO_4
III. Monomethylarsonic acid	MMAA	$CH_3AsO(OH)_2$
IV. Dimethylarsinic acid	DMAA	$(CH_3)_2AsO(OH)$
V. Trimethylarsine oxide	TMAO	$(CH_3)_3AsO$
VI. Tetramethylarsonium ion	Me_4As^+	$(CH_3)_4As^+$
VII. Arsenocholine	AC	$(CH_3)_3As^+CH_2CH_2OH$
VIII. Arsenobetaine	AB	$(CH_3)_3As^+CH_2COO^-$
IX. Dimethylarsinylethanol	DMAE	$CH_3)_2As(O)CH_2CH_2OH$

Arsenosugars



R:

X. -OH
XI. $-OPO_3HCH_2CH(OH)CH_2OH$
XII. $-SO_3H$
XIII. $-OSO_3H$

Figure 1.1: Major arsenosugars in marine algae

Inorganic forms of arsenic are generally much more toxic than the organic forms. For instance, animal data indicate that arsenite is more toxic than arsenate. Thus toxicity decreases in the order $As^{III} > As^{V} >$ MMAA > DMAA > AB[6, 7]. The mechanism of toxicity differs with the oxidation state of arsenic[6]. Arsenite has a high affinity for binding to thiol groups, which are present in the active centers of a number of enzymes. In the case of arsenate its toxicity is associated with its chemical resemblance to the phosphate ion. This mechanism uncouples oxidative phosphorylation and leads to the breakdown of energy metabolism[6,7].

On the other hand, the *in vivo* mechanism of detoxification in mammals involves methylation of inorganic arsenic to MMAA or DMAA[1]. As a result, arsenite, arsenate, MMAA and DMAA are commonly used as biomarkers for assessment of chronic exposure to arsenic[2].

In marine algae, arsenic is mainly found in organic forms, though some alga species have been reported to contain considerable amounts of inorganic arsenic[5]. Generally, arsenic concentrations in marine algae are considerably higher in brown algae than in either red or green algae[5].

Organoarsenicals may be water- or lipid-soluble. Arsenosugars are one example of water-soluble organoarsenicals. They are the main arsenic species in marine algae and are also found in some marine bivalves[4]. Most arsenosugars are dimethylarsinoylribosides, in which arsenic is bound to a carbohydrate molecule. Their molecular structures contain a common pentose moiety[5] (Figure 1.1).

The exact mechanism for arsenosugars biogenesis is yet to be elucidated[5]. However it is believed that algae may absorb arsenate because of its similarity to the phosphate anion. First, arsenate is methylated with S-adenosylmethionine (**SAM**, Figure 1.2) as the methyl donor. Then, another **SAM** molecule transfers its adenosyl group, forming the proposed intermediate **4** (Figure 1.3), which upon glycosidation with the common algal metabolites produces the range of arsenosugars observed in algae[4]. The proposed mechanism is shown in Figure 1.3.

Although arsenosugars are believed to be detoxification end products, their biochemical role is yet to be established[4]. It is commonly believed that arsenosugars pose no acute toxicity when ingested from seafood. However, their confirmed metabolism to DMAA in humans may have potential health effects for chronic exposure[8, 9].

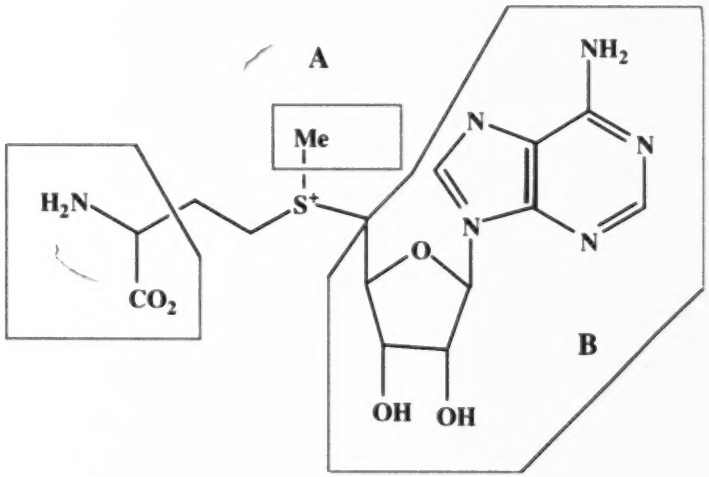


Figure 1.2: Structure of S-adenosylmethionine (SAM). A and B corresponds to the methyl and adenosyl groups, respectively

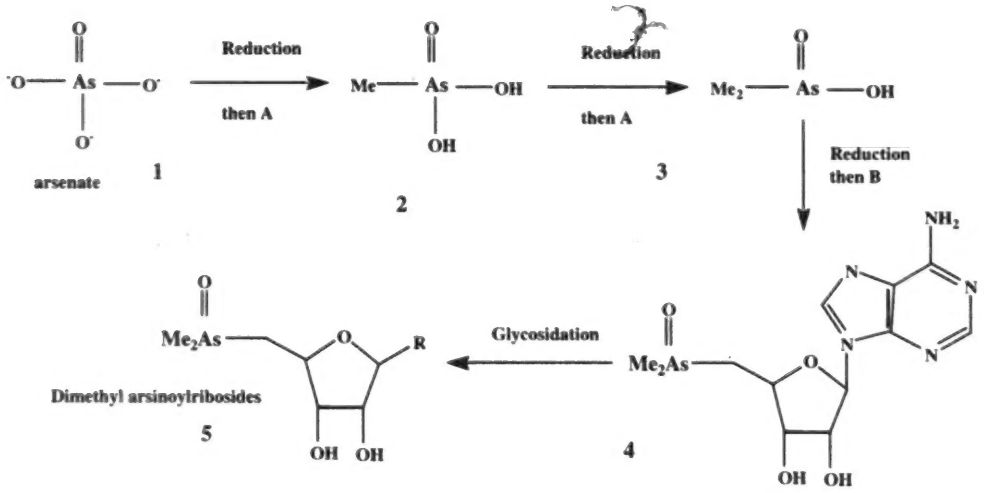


Figure 1.3: Mechanism for arsenosugars biogenesis in marine algae (A and B are shown in Figure 1.2)

Lipid –soluble arsenic also occur in algae, often as a major constituent. It has been shown that esterification of arsenosugars may lead to the formation of arsenolipids in marine and estuarine biota[2].

In marine animals, the major arsenic compound is arsenobetaine[5]. It was first isolated from the tail muscle of the western rock lobster in 1976 and has been shown to be present in virtually all-marine animals. Arsenobetaine is highly stable. In humans, ingested arsenobetaine is rapidly excreted unchanged in the urine. Although it has been suggested that arsenosugars may be involved in the production of arsenobetaine, its biogenesis in marine organisms is still unclear[4].

The tetramethylarsonium ion is also commonly found in marine animals, particularly in bivalve mollusks, where it can be the major species. Trimethylarsine oxide and arsenocholine also occur, generally as minor arsenic constituents, though exceptions have been reported[5].

1.2 ANALYTICAL METHODOLOGIES FOR ARSENIC SPECIATION

Both toxicity and bioavailability of arsenic are species-dependent[1,6]. Consequently, the determination of the total content of an analyte is not sufficient to assess toxicity, environmental impact or occupational exposure. Also, sensitive, reliable and rapid analytical techniques are needed to address the fact that individual species are usually present at lower concentrations.

Methods for identifying and quantifying arsenic species may be classified depending on the level of information they provide. First-level methods can distinguish between inorganic and organic arsenic. Second-level methods are capable of analyzing inorganic arsenic together with simple methylated species (MMAA, DMAA, and TMAO). Third-level methods extend the range to quaternary compounds and some other complex organoarsenicals, such as arsenobetaine, arsenocholine, and arsenosugars[5].

A commonly used first-level method[10] involves the reduction of the inorganic arsenic fraction to arsenic trichloride ($AsCl_3$) with potassium iodide (KI)/HCl. $AsCl_3$ is then separated from the reaction mixture by extraction with a non-polar solvent (usually toluene) or by distillation. The organic fraction is oxidized to As^V with acid mixtures (e.g. HNO_3/H_2SO_4) and determined by AAS with flame atomization (FAAS) or HG-AAS. The organic/inorganic ratio of arsenic can be calculated for the particular sample.

Spectrophotometric methods based on the formation of colored complexes between As^V and certain ligands are another example of first-level methods. In

this case[11], the sample is treated with an oxidizing agent like permanganate to convert the inorganic arsenic fraction to arsenate, which forms a blue complex with the molybdate ion. The absorbance of the complex is then measured at 865 nm. The detection limit of the method is 0.005 mg L^{-1}.

Second-level methods for arsenic species are generally based on hydride generation techniques (HG). Reducing inorganic arsenic and simple methylated species with $NaBH_4$ in acidic medium, produces characteristic volatile derivatives (arsines) which may be flushed from the sample matrix[5]. Arsines generated may be either thermally decomposed to elemental arsenic for atomic detection by atomic absorption spectrometry (AAS) or inductively-coupled plasma/atomic emission spectrometry (ICP-AES), or collected in a cold trap and then separated by fractional distillation or chromatography prior to arsenic-specific detection[12]. The hydride generation technique has the limitation that only inorganic arsenic or simple methylated derivatives are capable of arsine production upon direct reaction with $NaBH_4$[13].

Third-level methods combine chromatographic separation of the native arsenic compounds with arsenic-specific detection. High performance liquid chromatography (HPLC) with ion exchange or reversed phase columns is the most common separation method. HPLC has been coupled to AAS[14], atomic fluorescence spectrometry (AFS)[15], and AES[16] detectors. However, the best results in terms of sensitivity and detection limits are obtained when using mass spectrometry following decomposition of arsenic species and ionization to the ^{75}As ion by ICP with mass spectrometry (MS) detection[17].

The HPLC-ICP-MS system allows rapid separation and sensitive determination of arsenic species in crude marine extracts without pretreatment HPLC-ICP-MS can identify arsenic species in marine samples by comparison with retention times of appropriate standard compounds. However this system also has some limitations[18]. Structural information is lost during the analysis. Thus, unknown species cannot be identified. In terms of cost of purchase and operation, ICP-MS is one of the most expensive detectors for HPLC, posing restraints on its use in routine analysis.

Incorporating a decomposition/derivatization step after HPLC separation and prior to detection has allowed the use of cheaper and simpler detectors such as AAS[19] or AFS[15] for the analysis of crude marine extracts. Ultraviolet photolysis or microwave-assisted decomposition in the presence of potassium persulphate ($K_2S_2O_8$)/sodium hydroxide (NaOH) has been used to achieve complete decomposition of organoarsenicals to arsenate, which is then converted into arsine by the hydride generation technique. Detection limits are in the range of those found with the HPLC-ICP-MS system[19].

Recently, the potential of newer MS/MS techniques for structural information has started a new area of development in the field of elemental speciation. The application of the electrospray interface (ESI) for coupling HPLC or capillary electrophoresis (CE) to MS detectors promises to become a powerful system, capable of providing high separation efficiency as well as elemental (isotopic pattern) and structural information (molecular mass/fragmentation pattern)[20, 21, 22]. Several groups have reported the CE-ESI-MS[23, 24] or HPLC-ESI-MS[25, 26] analysis of standard solutions of arsenic compounds.

However, the use of non-volatile, ionic reagents in both CE and HPLC separations causes reduction in the electrospray stability and loss of sensitivity. As a result, in most cases these approaches have not proved useful for the analysis of real samples[23, 27].

Information of arsenic speciation from third-level methods can also be restricted by problems associated with extraction (solubilization). Ideally, an extraction procedure must be capable of quantitatively extracting the analyte from the sample while not altering the individual species in any way[5].

Mild polar solvents such as water/methanol, chloroform/methanol/water mixtures[28,29], are generally used to solubilize the arsenic species from seafood samples prior to their determination. However, this procedure may underestimate the presence of non-polar arsenic species. In general, the lack of well-established protocols for extraction of arsenic species makes the speciation analysis procedure-dependent.

Recent procedures[30] for extraction of arsenic species from environmental and biological samples involve using solvent at high temperatures and pressures (accelerated solvent extraction) or combining solvent extraction with techniques such as microwave-assisted extraction, enzyme-assisted extraction and ultrasonic extraction.

Accelerated solvent extraction uses liquid solvents at elevated temperatures and pressures to extract analytes from solid or slurry-like samples in very short periods of time and with small volumes of solvent[31].

This technique was compared with a standard sonication procedure for the extraction of arsenicals from fish tissue. Both methods were found to extract a similar distribution of arsenicals. However, the effect of high temperatures and pressures on the integrity of the arsenic species was not reported[32].

Microwave-assisted extraction is the process of heating solid sample–solvent mixtures with microwave energy and the subsequent partitioning of the compounds of interest from the sample to the solvent[30]. Ackley and co-workers[33] reported 100% of extraction efficiency of arsenic from fish tissue samples using microwave–assisted extraction. However, the applicability of this procedure to other arsenic species remains to be determined, as arsenic in fish tissue samples is essentially present only in the form of arsenobetaine.

Enzyme hydrolysis has been applied as a sample treatment for speciation purposes. Digestion with protease/lipases has been generally performed before extraction of organolead[34,35], organotin[36,37], organoselenium[38-40] and organoarsenic[41] compounds from seafood samples. In general, extractabilities of the enzyme–assisted approach have been comparable to the common techniques, and have preserved the original speciation of the sample under study.

Sonication is a popular sample preparation technique used with solvent extraction of solids[30]. The usefulness of sonication can be explained in terms of the phenomenon of cavitation.

Cavitation involves the production and explosion of countless microbubbles or cavities in liquids, upon the application of ultrasound energy at a

rate 20-40 kHz. Ultrasound energy generates alternating high and low pressure waves which compress and expand the liquid. As a result, the microbubbles grow until a maximum critical diameter and then explode[30].

The repetition of the formation-implosion cycle all over the liquid body produces vigorous agitation at the surface of the solid being extracted. The ultimate effect is the release of analytes from the solid surface[42]. The extractive power is enhanced by the generation of high effective temperatures and pressures at the solid-liquid interface, as well as by the production of hydroxyl radicals and hydrogen peroxide upon sonolysis of water[42].

Several factors influence the efficiency of cavitation. Frequency and amplitude of the ultrasound wave will determine how much irradiation power is transmitted from the probe into the liquid under sonication. Viscosity, density, vapor pressure and temperature will also influence the dissipation efficiency of ultrasound power within the liquid under sonication[30].

Ultrasonic-assisted extraction has been applied to speciation of chromium[43], cadmium[44], mercury[45] and lead[46] in a number of environmental and biological samples. Ultrasound treatment of the samples combined with acidic extraction assured the quantitative extraction of these analytes in most cases.

1.3 RESEARCH OBJECTIVES

The overall objective of this thesis was to study the effect of several sample treatments on the arsenosugars present in commercial seaweed products.

In Chapter 2, seaweed extracts were treated under several digestion treatments and analyzed for arsenic by FI-HG-AAS. Arsenosugars decomposition to hydride-forming species (HFS) was determined by the ratio of arsenic concentrations in each extract before and after microwave-assisted digestion with $K_2S_2O_8$/NaOH. It was found that arsenosugars were not decomposed to hydride-forming species by simulated cooking or *in vitro* gastrointestinal digestion.

Chapter 3 and 4 reported the use of enzyme-assisted and ultrasonic-assisted extractions to improve the extraction efficiency of arsenic from seaweed. Although the enzymatic treatment did not improve the extraction efficiency *per se* under the present experimental conditions, the sequential extraction approaches tested on seaweed samples (bath/probe sonication, sonication/enzyme hydrolysis) yielded extraction efficiencies comparable to those by the conventional procedure (water/methanol extraction with bath sonication). Both treatments did not decompose the native arsenic species in the seaweed samples as shown by FI-HG-AAS and HPLC-ICP-MS analyses. Also, the performance of the extraction procedures seemed to be strongly dependent on sample homogeneity.

CHAPTER 2

STABILITY OF ARSENOSUGARS UNDER DIFFERENT SAMPLE TREATMENTS

2.1 INTRODUCTION

To obtain meaningful results in speciation analysis, it is crucial to preserve the integrity of the sample and the species of interest between the time of collection and the opportunity for analysis[47]. Consequently, special care must be taken to plan and develop protocols for sample collection, sample storage and pretreatment[2]. However, preserving the original speciation in a sample may not be an easy task as many species are thermodynamically unstable[48] and treatments such as dissolution, extraction and preconcentration may shift their equilibria, even destroy them or transform them into another species[47].

Also, as the concentration of the individual species is much lower than the total concentration of the analyte, an enrichment step is normally required. As a result, introducing additional steps in the analytical scheme may lead to a higher risk of sample contamination and increased analyte losses[48].

In the field of arsenic speciation, little attention has been paid to preserving the distribution of arsenic species during the time between collection

and analysis[2]. For instance, it has been reported that arsenosugars are quite unstable under extreme pH conditions, which makes them difficult to purify, characterize and preserve. Arsenosugars are mainly decomposed to DMAA in strongly acidic or basic media[49], though, it is quite likely that other degradation products may be formed.

Also, the conversion between arsenosugars in fresh algae samples has been reported [50] In this case, an enzymatic mechanism has been suggested to operate[50]. However, no confirmatory data justifying this has been published so far.

Human ingestion of arsenosugars-containing seaweed and mussels has resulted in the urinary excretion of its metabolites, not the original arsenosugars[8,9].To distinguish human metabolism of arsenosugars from simple decomposition, it is important to demonstrate that arsenosugars are not decomposed during cooking or by gastrointestinal fluid.

The objective of this study was to investigate the effect of some sample treatments on arsenosugars stability. Arsenic concentrations in seaweed samples were determined by FI-HG-AAS measurements with or without microwave–assisted decomposition with $K_2S_2O_8$/NaOH. Arsenosugars stability was studied by determining the percent hydride-forming species (HFS), which is the ratio of arsenic concentration in the digested extract divided by the total arsenic content in the extract by microwave–assisted decomposition with $K_2S_2O_8$/NaOH. Results were used to determine the extent of arsenosugars decomposition to hydride-forming species under these treatments.

2.2 EXPERIMENTAL

2.2.1 INSTRUMENTATION

An atomic absorption spectrophotometer (SpectrAA-5, Varian) was used for detection of arsenic. The FI-HG-AAS system is schematically shown in Figure 2.1. An arsenic hollow cathode lamp (193.7 nm) was operated at 10 mA using an external control module (Varian). The spectral bandwidth was 0.5 nm. A T-shaped quartz absorption tube (Varian) heated to 925°C with a temperature controller module (module ETC-60, Varian) was used as the atomization cell. A computer with a Varian Star Workstation ADC board and software was used to record and process the atomic absorption signals.

Basically, the FI-HG-AAS system consisted of a four-channel peristaltic pump (Minipuls 3, Gilson) with adjustable speed for the delivery of reagent solutions and sample carrier (deionized water). Sample injection was accomplished by means of a six-port sample injection valve (Rheodyne) with a 500-µL sample loop. The injected sample was carried by a continuous flow of deionized water (3 mL/min) and met with continuous streams of hydrochloric acid (1.0 M HCl, 5 mL/min) and reducing agent (0.6 M $NaBH_4$, 5 mL/min) at two T-joints. Arsines were generated and separated from liquid waste in a gas-liquid separator. The gaseous arsines were then swept by a continuous flow of argon gas (100 mL/min) to the atomic absorption spectrometer. Hydride generation conditions were adapted from results published elsewhere[51].

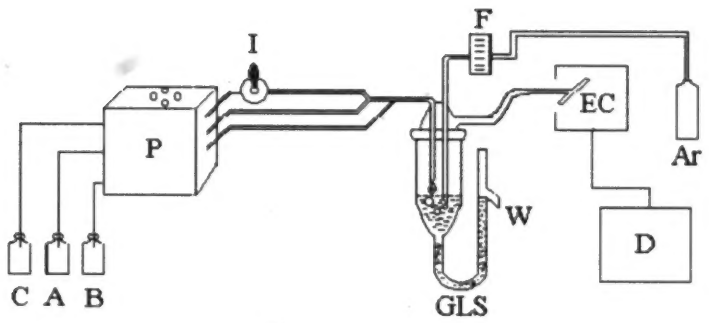


Figure 2.1: Schematics of the FI-HG-AAS system (adapted from Reference 51)

A: HCl solution
B: $NaBH_4$ solution
C: deionized water carrier
P: Peristaltic pump
I: Sample injection valve
GLS: Gas-liquid separator
EC: Electrically heated quartz cell
Ar: Argon gas
F: Flowmeter
W: Waste
D: Atomic absorption detector

2.2.2 HYDRIDE GENERATION REACTIONS[52]

$NaBH_4$ is commonly used for converting arsenic species in aqueous solutions into volatile hydrides. This reaction is believed to proceed by a two-step mechanism. The first step corresponds to the reduction of the arsenic species to the +3 state as follows:

$$R_nAs(O)(OH)_{3-n} + H^+ + BH_4^- \longrightarrow R_nAs(OH)_{3-n} + H_2O + BH_3$$

In most current speciation studies, **R** is methyl group and **n** ranges from 0 to 3. Additional $NaBH_4$ further reduces the arsenic compound to its corresponding arsine:

$$R_nAs(OH)_{3-n} + (3- n)\ H^+ + (3- n)\ BH_4^- \longrightarrow R_nAsH_{3-n} + (3- n)\ BH_3 + (3- n)\ H_2O$$

Hydrolysis of borane generated by these reactions yields boric acid and gaseous hydrogen:

$$BH_3 + 3H_2O \longrightarrow H_3BO_3 + 3H_2$$

2.2.3 STANDARDS AND REAGENTS

Deionized water from a Milli-Q ultrapure water system (Millipore) was used for the preparation and dilution of reagents, samples, and calibration solutions. Stock solutions of arsenic (1000 mg L^{-1}) were prepared by dissolving sodium arsenite ($NaAsO_2$, Sigma), sodium arsenate (Na_2HAsO_4, Sigma),

dimethylarsinic acid ($C_2H_7AsO_2$, Sigma) and monosodium methane arsonate ($NaCH_4AsO_3$, Chem. Service) in deionized water. Calibration solutions were then prepared by diluting the stock solution.

Calibration solutions containing more than 1 mg L^{-1} of arsenic species were stable for several months. As arsenate can be reduced to arsenite in aqueous solutions calibration solutions containing less than 10 μg L^{-1} arsenic were prepared fresh daily by serial dilution with deionized water from 1 mg L^{-1} arsenic calibration solution. Arsenic concentration in the stock solution was calibrated against an atomic absorption arsenic standard solution containing 1000 mg L^{-1} of arsenic (Aldrich) using both ICP-MS (VG Plasma Quad Turbo Plus, VG Elemental, Fison) and flame atomic absorption spectrometry (SpectrAA-200, Varian).

$NaBH_4$ (Aldrich) and $K_2S_2O_8$ (Fisher) solutions in NaOH (0.1 M, BDH) were prepared fresh daily. HCl, sulphuric acid (H_2SO_4), nitric acid (HNO_3), and sodium bicarbonate ($NaHCO_3$) were purchased from BDH. Tetraethylammonium hydroxide (TEAH) and methanol (CH_3OH) were purchased from Aldrich and EM Science respectively. All reagents were of analytical grade or better.

Pepsin (P-7000), pancreatin (P-1750), α-amylase (A-6880) and bile salts (B-8756) were purchased from Sigma and used without further purification.

2.2.4 SAMPLES

The commercial seaweed products, powdered kelp (brown algae) and Yakinori (red algae) were purchased from a local food store (Edmonton, Canada). Yakinori was in the form of thin sheets.

Duplicate samples of both kelp and Yakinori (100 mg) were weighed in 50-mL centrifuge tubes and mixed with 10 mL of methanol-water (1:1, v/v). Each sample was then sonicated in an ultrasonic bath for 10 minutes. After centrifugation the supernatant was removed and placed in a 100 mL beaker. The extraction process with the aid of sonication was repeated a further four times for each sample. The supernatants were combined in the beaker, evaporated until near dry and reconstituted to 20 mL with deionized water.

2.2.5 PROCEDURES

2.2.5.1 Digestion of seaweed extracts under different chemical treatments

Reagents and their corresponding concentrations are listed in Table 2.1. Briefly, an aliquot of each sample (1 mL) was placed in a 100-mL beaker, combined with the digestion reagent and diluted to approximately 10 mL with deionized water.

Each beaker was covered by a watch glass and gently heated for about 30 minutes on a hot plate. After heating, the samples were cooled to room temperature, neutralized and quantitatively diluted to 10 mL with deionized water.

2.2.5.2 Digestion of kelp extracts under *in vitro* gastrointestinal conditions

Following a procedure reported elsewhere[53], triplicate samples of kelp (1 ± 0.0001g) were weighed in 50-mL centrifuge tubes and mixed with 10 mL of synthetic gastric juice (1% w/v pepsin in 0.15 M NaCl acidified with HCl to pH 2.0). Samples were then incubated for 2 hours at 37°C with occasional agitation. After adjusting the pH to 5.0 with saturated $NaHCO_3$, 10-mL synthetic gastrointestinal juice (1.5% pancreatin, 0.5% amylase and 0.15% bile salts, w/v, in 0.15 M NaCl) was added. The pH was then adjusted to 7.0 and the samples incubated for a further 2 hours at 37°C. Reagent blanks were taken through the entire procedure. At the end of the gastrointestinal stage, samples were removed and centrifuged for 20 minutes at 2500 rpm. Supernatants were decanted in 50-mL centrifuge tubes, diluted to 20 mL with deionized water and stored in a refrigerator (+4°C) until required for analysis.

2.2.5.3 FI-HG-AAS Analysis

Digested samples were directly analyzed for arsenic by using FI-HG-AAS. A 500-µL sample was injected into a deionized water carrier stream by a sample injection valve and hydride generation took place as described in Section 2.2.1.

Peak areas of atomic absorption (AA) signals were measured and standard arsenate solutions were used for calibration. Blank solutions were also prepared in the same way as samples and standards, and used for signal correction.

2.2.5.4 HPLC-ICP MS Analysis

When required, sub-samples of seaweed extracts (undigested and digested) were sent to Dr W.R. Cullen's Research Group, Department of Chemistry, University of British Columbia (Vancouver, Canada). HPLC-ICP-MS analysis of the extracts were kindly performed by Ms. V. Lai under the experimental conditions reported in Reference 54.

Table 2.1: Digestion treatments for seaweed samples

*TREATMENT	REAGENT	BOILING (min)
A. Dilution	9 mL deionized H_2O	NO
B. Simulated cooking	9 mL deionized H_2O	YES, 30
C. Mild acid digestion	1 mL, 1.0 M HCl	YES, 30
D. ^Gastrointestinal digestion	10 ml , gastrointestinal fluid	NO
E. Basic digestion	1 mL, 1.0M NaOH	YES, 30
F. TEAH digestion	0.1 mL, 1.0 M TEAH	YES, 30
G. TEAH digestion	1 mL, 1.0 M TEAH	YES, 30
H. Strong acid digestion	1 mL, [HNO_3/H_2SO_4] (3:1)	YES, 30
I. Total digestion	0.3M $K_2S_2O_8$/0.6 M NaOH	#MW, 30

* A 1.0-mL aliquot of the seaweed extract prepared in Section 2.2.3 was used in each treatment

^ Prepared from a separate experiment under Section 2.2.4.1 in this Chapter

\# Microwave-assisted decomposition with $K_2S_2O_8$/NaOH

2.3 RESULTS AND DISCUSSION

2.3.1 CHOICE OF HYDRIDE GENERATION CONDITIONS

The hydride generation reaction depends on the pH of the reaction medium[52]. Prior to any sample analysis, it was necessary to study the behavior of typical arsenic species, such as arsenite, arsenate, MMAA and DMAA, under the present experimental conditions.

From Figure 2.2, it is seen that maximum responses for hydride generation of arsenite, arsenate, MMAA and DMAA occurred at different HCl concentrations, a behavior associated with the acid-base properties of the individual species in solution. In agreement with previous reports[55], arsenite and arsenate required strongly acidic conditions ($[H^+] \geq 3M$) for maximum response. DMAA and MMAA were better determined with acid concentrations in the range 0.5-1.5 M. As a result, it was not possible to choose an optimized HCl concentration in order to yield maximum responses for all the species.

A compromised HCl concentration (1.0 M), which yielded approximately the same response for the arsenic species evaluated, was then chosen. Other HG experimental conditions have been presented in Section 2.2.1.

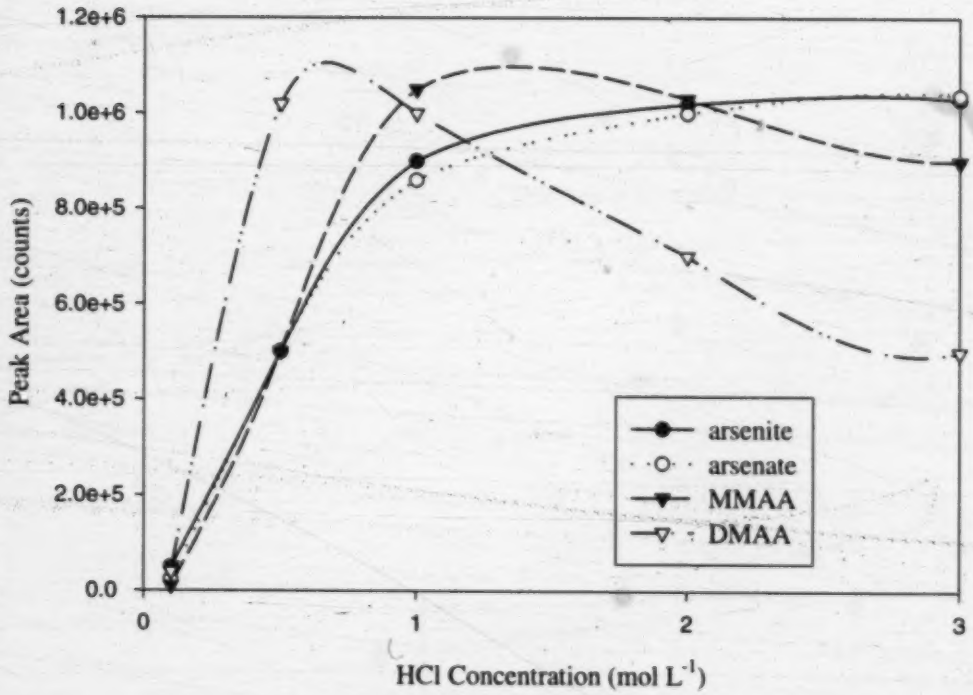


Figure 2.2 Effect of hydrochloric acid on hydride generation responses from 20 ng mL^{-1} of arsenite, arsenate, MMAA and DMAA

2.3.2. HPLC ANALYSIS OF ARSENIC-CONTAINING SAMPLES

Figures 2.3-5 show chromatograms obtained from a standard solution of arsenic species, a standard reference material (oyster tissue) and kelp powder, respectively.

As expected, arsenosugars were the major arsenic species in kelp (peaks X-XIII Figure 2.5). Structures were already described in Figure 1.1 in Section 1.1, Chapter 1. By comparing chromatograms in Figures 2.3 and 2.5, the humps, which appeared at 300-400 seconds (Figure 2.5) might be identified as arsenate and DMAA. Arsenobetaine was the major arsenic compound in oyster tissue (Figure 2.4), which also contained two arsenosugars and DMAA.

2.3.3 ARSENIC DETERMINATION IN SEAWEED EXTRACTS BY FI-HG-AAS

For each digestion treatment, the concentration of arsenic in the sample was determined by FI-HG-AAS, using a calibration curve prepared with standard solutions of arsenic (Figures A1 and A2 in Appendix A, data shown in Tables B1 and B3 in Appendix B).

The percent HFS was calculated as the ratio of arsenic concentration in the digested extract (treatments A-H, Table 2.1) divided by the arsenic concentration in the extract after microwave-assisted decomposition with $K_2S_2O_8$/NaOH (treatment I, Table 1.2).

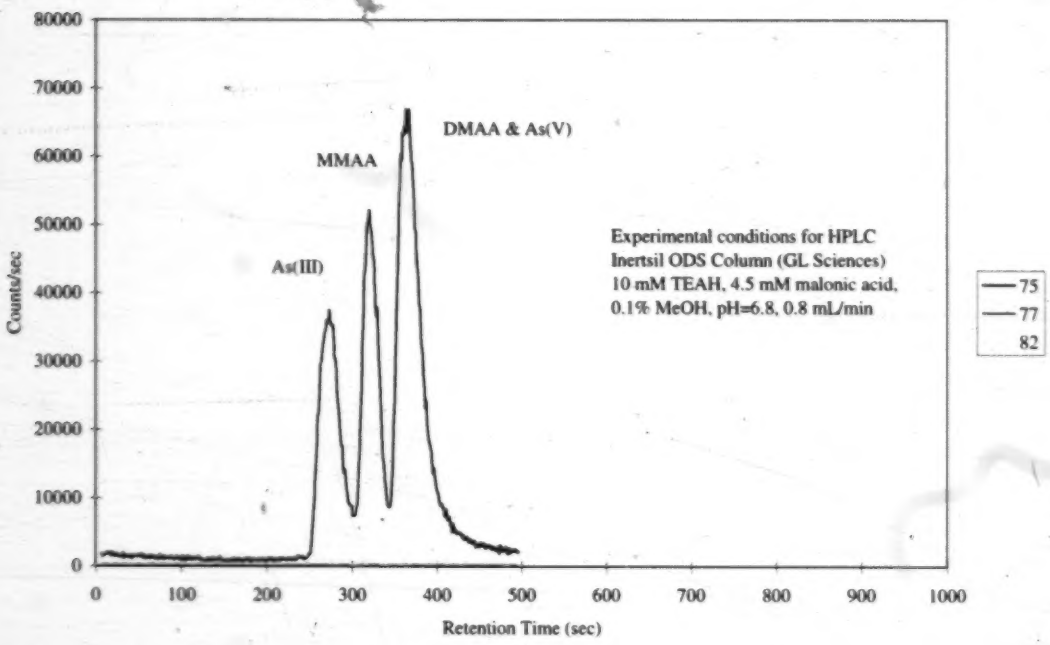


Figure 2.3: Chromatogram obtained from a standard solution of arsenite, arsenate, MMAA and DMAA (100 μg L^{-1} each). Detection was performed by ICP-MS. Other experimental conditions were described in Ref. 54 Arsenic species were labeled according to Table 1.1 in Section 1.1, Chapter 1

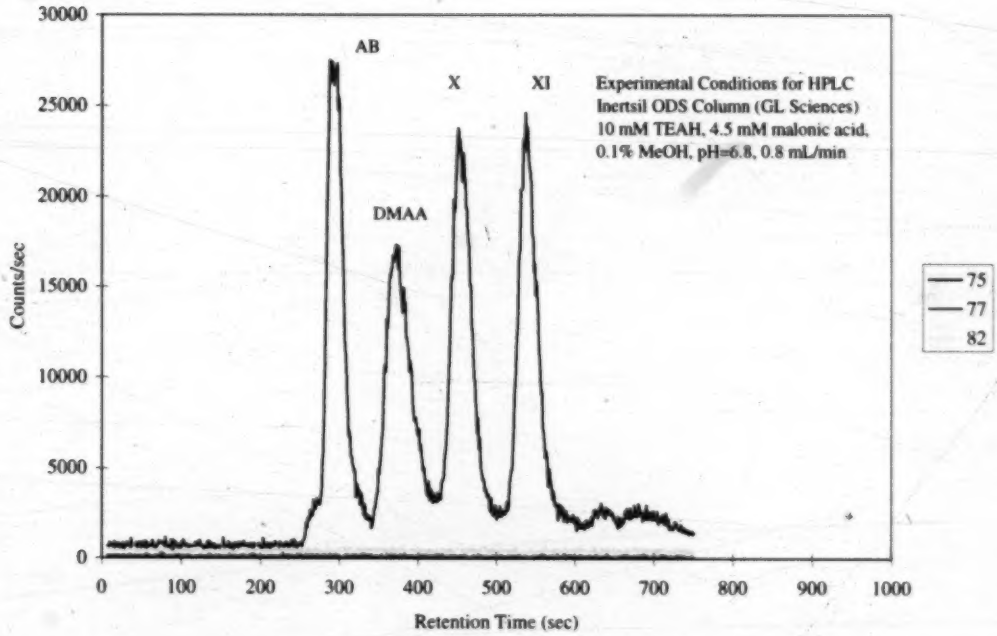


Figure 2.4: Chromatogram obtained from an extract of oyster tissue (SRM NIST 1566a, National Institute of Standards and Technology, US Department of Commerce). Detection was performed by ICP-MS. Other experimental conditions were described in Ref. 54. Arsenic species were labeled according to Table 1.1 and Figure in Section 1.1, Chapter 1. Peaks X-XI corresponded to arsenosugars

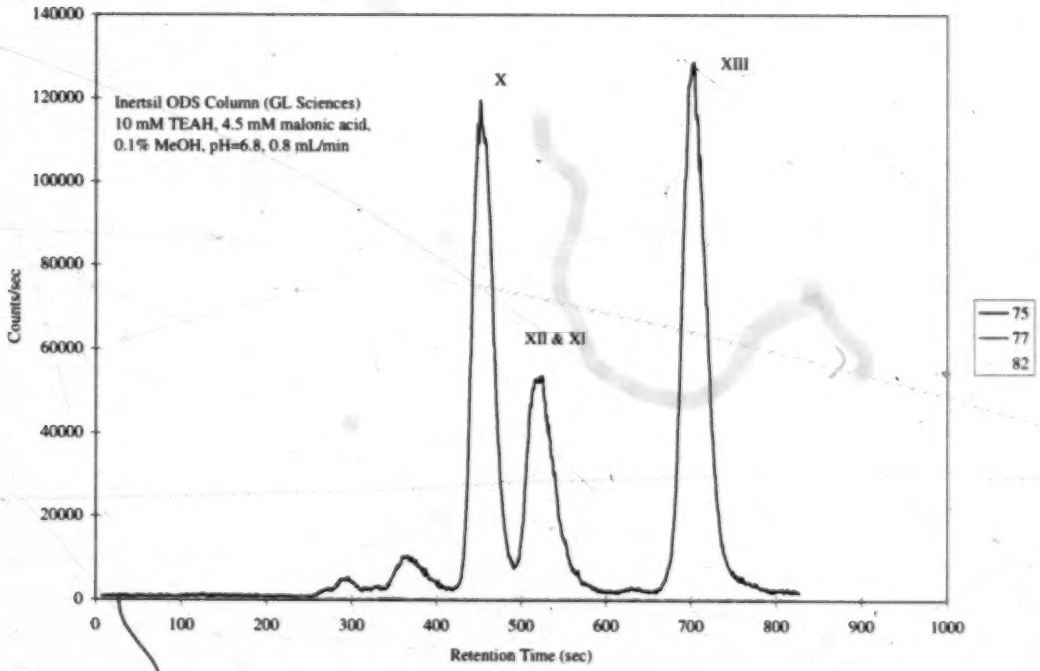


Figure 2.5: Chromatogram obtained from kelp powder (commercial seaweed product). Detection was performed by ICP-MS. Other experimental conditions were described in Ref. 54. Arsenic species were labeled according to Table 1.1 and Figure 1.1 in Section 1.1, Chapter 1. Peaks X-XI corresponded to arsenosugars

As mentioned previously, arsenosugars do not produce volatile arsines upon direct reaction with $NaBH_4$ Consequently, any analytical response towards hydride generation should be due either to the decomposition of arsenosugars to hydride-forming species, or to the presence of hydride-forming species (inorganic and methylated forms of arsenic).

Figures 2.6-2.8 show typical AAS responses from arsenic standards, and kelp and Yakinori extracts. Results for the percent HFS of digested kelp and Yakinori extracts are shown in Figures 2.9 and 2.10 respectively. Arsenic concentrations in µg of arsenic per gram of dry sample are shown in Tables B2 and B4 in Appendix B.

2.3.3.1 Arsenic determination in undigested seaweed extracts

The percent HFS amounted to 5.5% and 15% in kelp and Yakinori extracts respectively. In both cases, results from HPLC-ICP-MS and FI-HG-AAS confirmed the presence of hydride-forming species. The HFS value reported in the literature in Yakinori samples was 2-3% of the total arsenic content (as DMAA and arsenate[50]). The difference between the present and reported values may well be explained in terms of algae types used to prepare the commercial product. Furthermore, algae harvested at different locations and/or seasons may have different distribution and concentration of arsenic species[50].

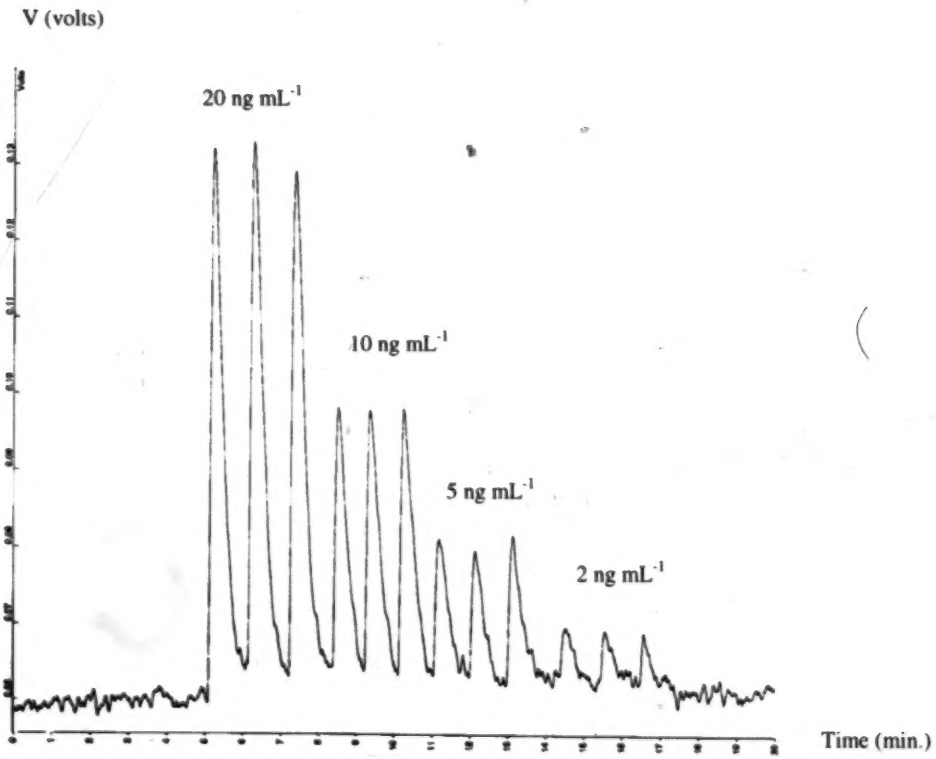


Figure 2.6: Typical signals obtained from 500 µL of 2, 5, 10 and 20 ng mL^{-1} of As^{V} by using FI-HG-AAS. Curve was linear in the present concentration range. RSD values typically ranged from 2-5%

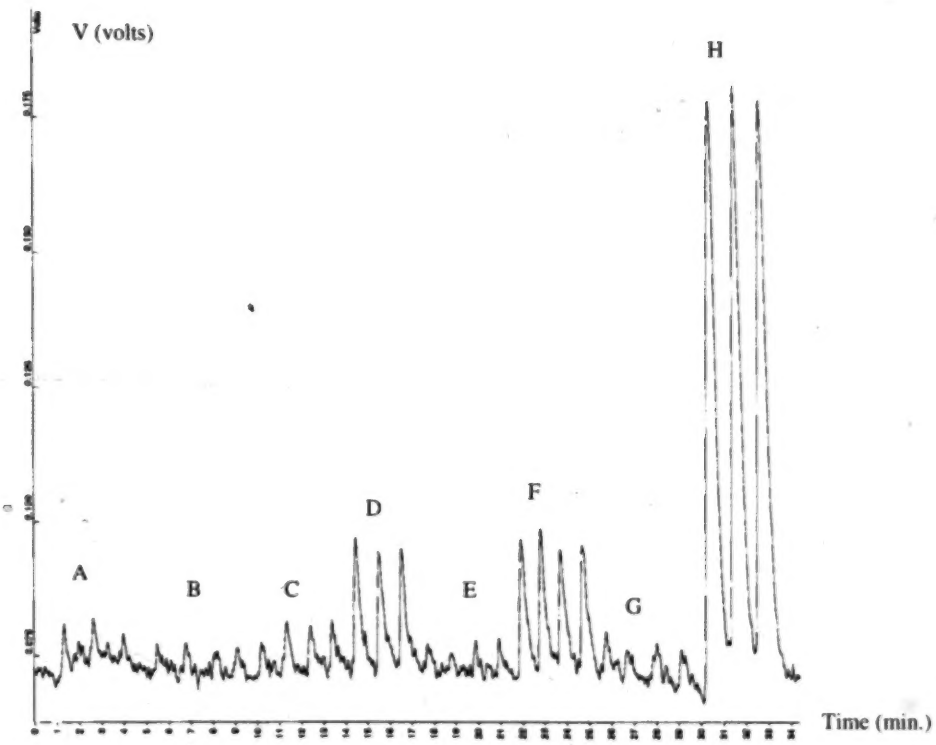


Figure 2.7: FI-HG-AAS signals of arsenic in kelp extracts after different digestion treatments. A: Diluted extract; B: Simulated cooking; C: 0.1 M HCl digestion; D: 0.1 M NaOH digestion; E: 0.01 M TEAH digestion; F: 0.1 M TEAH digestion; G: H_2SO_4/HNO_3 digestion; I: Microwave-assisted digestion with $K_2S_2O_8$/NaOH (procedure as in Sections 2.2.5.1-2)

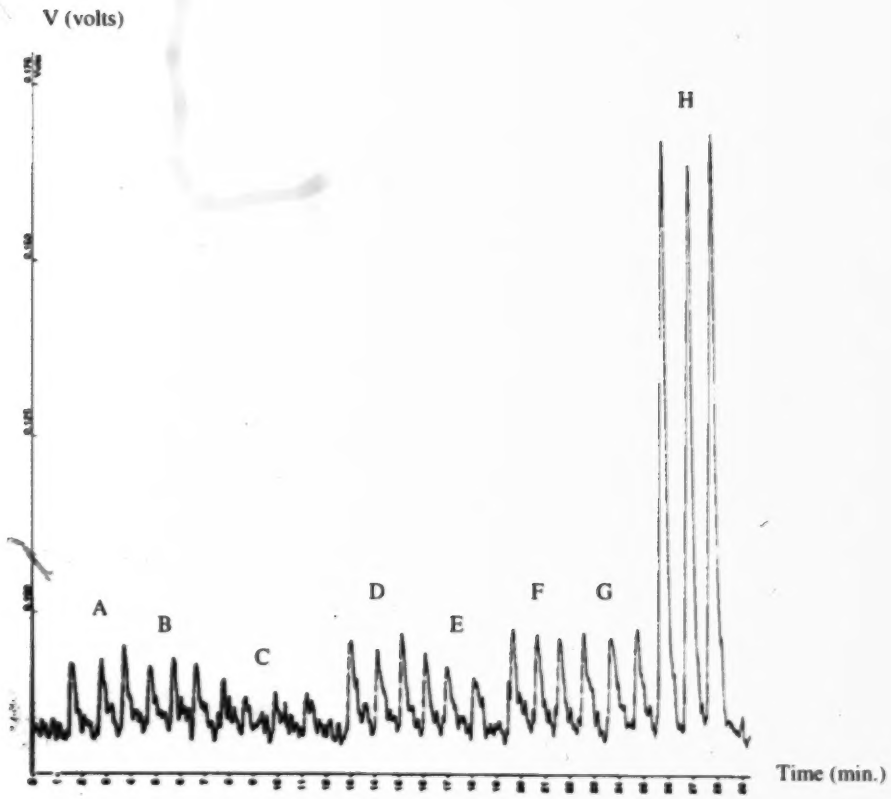


Figure 2.8: FI-HG-AAS signals of arsenic in Yakinori extracts after different digestion treatments. A: Diluted extract; B: Simulated cooking; C: 0.1 M HCl digestion; D: 0.1 M NaOH digestion; E: 0.01 M TEAH digestion; F: 0.1 M TEAH digestion; G: H_2SO_4/HNO_3 digestion; I: Microwave-assisted digestion with $K_2S_2O_8$/NaOH (procedure as in Sections 2.2.5.1)

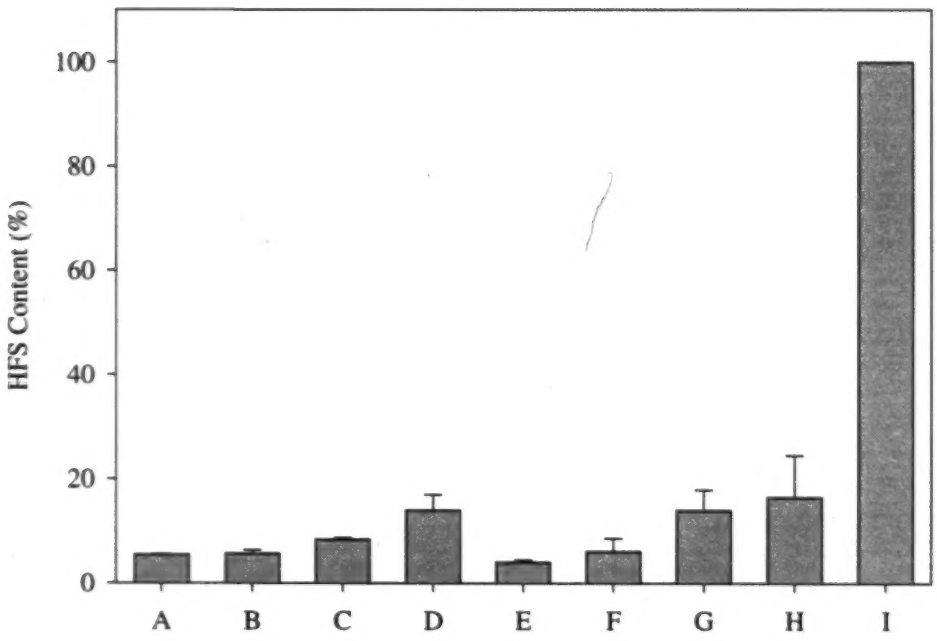


Figure 2.9: Arsenic content in kelp samples after different digestion treatments (n=2). Labels and treatments were described in Table 2.1 and Section 2.2.5.1-2

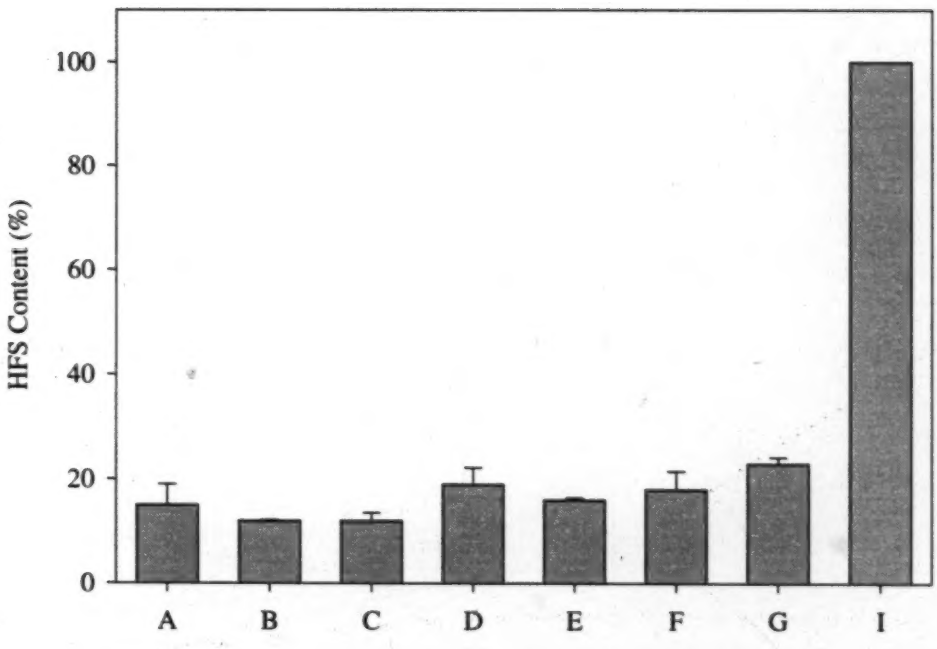


Figure 2.10: Arsenic content in Yakinori samples after different digestion treatments (n=2). Labels and treatments were described in Table 2.1 and Sections 2.2.5.1

2.3.3.2 Arsenic determination in seaweed extracts after simulated cooking conditions

It was thought that the cooking procedure might decompose the arsenic species present in the seaweed samples prior to ingestion. This was studied by gently boiling the seaweed samples in water for 30 minutes. The percent HFS in kelp and Yakinori samples amounted to 5.7 and 12 %, respectively. Differences in HFS contents were not significant when compared to those of the undigested samples. These results suggested that arsenosugars were not substantially decomposed by the cooking procedure performed on the seaweed samples under the present experimental conditions.

From Figure 2.11, it is seen the profile of the "cooked" extract (b) is essentially the same when compared to that of the undigested extract (a). Neither retention times nor were relative intensity of arsenosugars peaks changed.

2.3.3.3 Arsenic determination in seaweed extracts after digestion with acid reagents

The influence of acid reagents on arsenosugars decomposition was investigated by gently boiling the seaweed extracts in the presence of 0.1 M HCl and HNO_3/H_2SO_4 for about 30 minutes.

The percent HFS after boiling the samples in 0.1M HCl was 8.4 and 12% for kelp and Yakinori samples respectively. In both cases, differences with respect to the undigested samples were not significant.

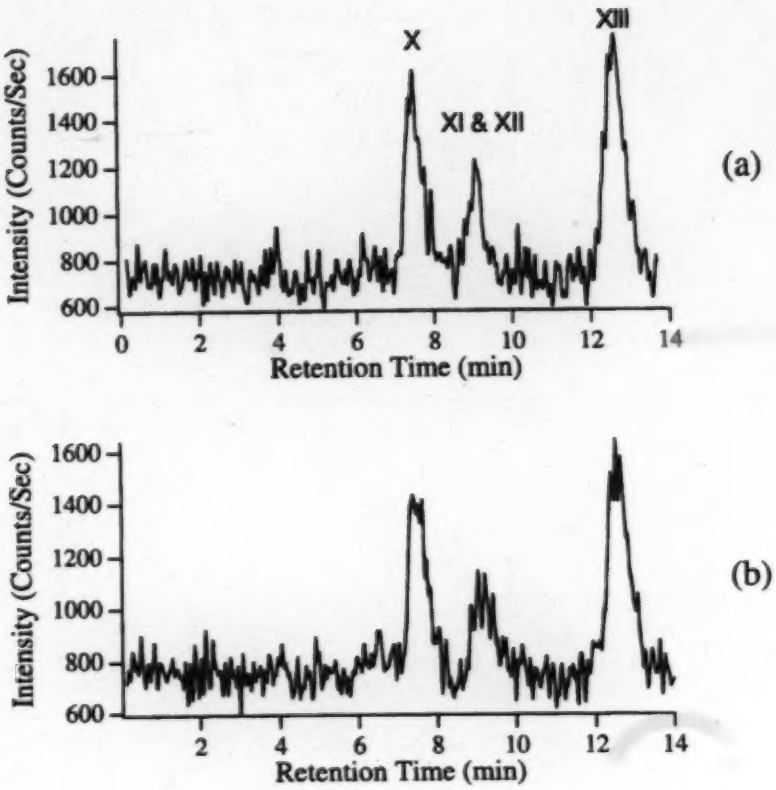


Figure 2.11: Chromatograms obtained from HPLC-ICP-MS analysis of a undigested kelp extract (a) and a "cooked" kelp extract (b). HPLC conditions were as in Figures 2.3-5 in Section 2.3.2 in this Chapter. Peaks X-XIII corresponded to arsenosugars (structures shown in Figure 1.1 in Section 1.1, Chapter 1)

As in the preceding case, the simulated gastric digestion treatment with 0.1M HCl did not seem to be involved in the decomposition of arsenosugars to arsines-forming species under the present experimental conditions. Le and co-workers[8] reported a similar result in Nori samples (a commercial red-algae product).

When the kelp extracts were boiled in HNO_3/H_2SO_4, the corresponding percent HFS amounted to 16% for kelp and 23% for Yakinori. A mixture of HNO_3/H_2SO_4 is usually employed for destruction of the organic matrix in the samples and complete conversion of arsenic species into arsenate. However, differences with respect to the HFS percent in undigested extracts were not significant. Complete decomposition of arsenosugars usually requires longer digestion time (2-6 hours). In this case, samples were only boiled for 30 minutes in a minimal amount of acid.

2.3.3.4 Arsenic determination in seaweed extracts after in vitro gastrointestinal digestion

In a separate experiment, only kelp extracts were digested under these conditions (under procedure in Section 2.2.5.2 in this Chapter). Arsenic concentrations were determined by using the calibration curve shown in Figure A3 in Appendix A, data shown in Table B5 in Appendix B.

Results are summarized in Table B6. The HFS percent in the samples after gastrointestinal digestion (enzyme-digested) amounted to 4.1 ± 0.4 % (n=3). In the control samples (non enzyme-digested), the HFS percent was 5.1 ± 0.4 %

(n=3). Again, differences were not significant and agreed with previous results of undigested extracts from treatment A (Table 2.1, Section 2.2.4.1 in this Chapter) Consequently, digestive enzymes did not decompose arsenosugars to hydride-forming species under the present experimental conditions.

2.3.3.5 Arsenic determination in seaweed extracts after digestion with basic reagents

The effect of a basic medium on the decomposition of arsenosugars was also investigated by gently boiling the seaweed extracts in 0.1M NaOH, 0.01M TEAH and 0.1M TEAH for 30 minutes.

Digestion with 0.1M NaOH yielded a HFS content of 14 and 19 % for kelp and Yakinori samples respectively. Digestion with 0.01M TEAH produced a HFS content of 6.1 and 16 % for kelp and Yakinori samples respectively. Increasing the concentration of TEAH up to 0.1 M yielded 14 and 18 % of HFS in kelp and Yakinori samples, respectively.

As TEAH is commonly used for ion pair HPLC separation of arsenosugars at elevated temperature (70 °C), it was thought that TEAH-containing mobile phases might be involved in the decomposition of arsenosugars upon HPLC analysis of seaweed samples. However, the HFS content in kelp and Yakinori extracts after TEAH digestion was not significantly different when compared to that of non-digested samples.

2.3.2.6 Arsenic determination in seaweed extracts after microwave-assisted decomposition with $K_2S_2O_8$/NaOH

Organoarsenicals, such as arsenosugars and arsenobetaine, are resistant to hydride generation upon reaction with sodium borohydride[6]. When these compounds are subjected to microwave-assisted decomposition with $K_2S_2O_8$/NaOH, complete decomposition to arsenate is achieved as previously reported[13]. Arsenate is then reduced to arsine by reaction with $NaBH_4$. Arsenic signals are roughly 8 and 18 times higher than those after the other treatments, as shown in Figures 2.7 and 2.8. As expected, digestion with persulphate in basic medium and microwave decomposition was the only treatment capable of totally converting arsenosugars to arsenate.

In summary, results from the FI-HG-AAS and HPLC-ICP-MS analysis of seaweed samples did not seem to implicate conditions such as cooking, mild acid digestion, gastrointestinal digestion or HPLC separation in the decomposition of arsenosugars to hydride-forming species. Consequently, the reported metabolism of arsenosugars in humans[8,9] is unlikely due to their decomposition prior to ingestion of arsenosugars-containing seafood.

When arsenosugars were digested under extreme pH conditions (treatment D, F and G, Table 2.1 in Section 2.2.5.1), the percent HFS did not indicate substantial decomposition to hydride-forming species. These results were not in agreement with those reported by others[49,50].

CHAPTER 3

ENZYME HYDROLYSIS OF SEAWEED SAMPLES FOR ARSENIC SPECIATION

3.1 INTRODUCTION

The majority of arsenic species present in seaweed are water-soluble and thus polar solvents such as water or water/methanol mixtures are commonly used to solubilize the arsenic species prior to their determination[5]. However, extraction efficiencies are generally low. In some cases, up to 70 % of the arsenic content remains unextracted[54]. Although it has been suggested that the residual fraction might be in the form of lipid-soluble arsenic species or more polar arsenosugars, little is known as to the nature of this residual arsenic. In this context, low extraction efficiencies may reflect the inadequacy of the extraction procedure[5].

It is also thought that the presence of cell wall polysaccharides limits the extractabilities by preventing the solvent from solubilizing the arsenic species trapped in the cell walls, either physico-mechanically or chemically as coordination complexes with plant cell components[40,57]. A similar reason has been invoked to explain low protein extractabilities from seaweed[58].

As a result, research on elemental speciation of the water insoluble fraction after extraction of plant samples has been challenged by difficulties

associated with the selective destruction of the residual organic matrix without altering the analyte of interest [57]. It is worth mentioning the report by Casiot[40] and co-workers on the use of a commercial enzyme preparation to destroy yeast cell walls for organoselenium speciation. Enzyme digestion as part of a sequential procedure involving leaching with hot water and sodium dodecyl sulphate proved useful for obtaining speciation information without the need for a hyphenated technique[40]. Also, Spuznar and co-workers[57] used a commercial enzyme preparation containing high levels of pectinolytic activities to release the metal-carbohydrates complexes present in fruit and vegetable homogenates. The extent of the release depended on the analyte and the type of sample. In most cases, the residual analyte content ranged from 0.5-5.8% (in mass percent of the initial homogenate).

As a way to improve the extraction efficiencies of arsenic species from seaweed samples, the objective of this study was to investigate the feasibility of enzyme hydrolysis of the cell wall polysaccharides in order to gain access to the residual fraction of the analyte in the sample.

In plant samples, the cell wall matrix is generally composed of pectic polysaccharides, hemicellulose and cellulose[59]. Therefore, enzyme preparations containing cellulase/hemicellulase cellulase/pectinase and lysing enzymes were tested for hydrolytic activity on seaweed samples. This hydrolytic activity was indirectly measured by determining the extraction efficiency of each enzymatic treatment.

3.2 EXPERIMENTAL

3.2.1 INSTRUMENTATION

An atomic absorption spectrophotometer (Varian, SpectrAA-5) was used for detection of arsenic. The FI-HG-AAS system has been described in Section 2.2.1.

3.2.2 STANDARDS AND REAGENTS

Deionized water from a Milli-Q ultrapure water system (Millipore) was used for the preparation and dilution of all reagents, samples, and calibration standards as described in Section 2.2.3. Na_2HAsO_4, (Sigma) was used to prepare arsenic calibration standards.

Cellulase (C-9422), hemicellulase (H-7649), pectinase (P-2401), and lysing enzymes (L-2265, containing cellulase, protease and chitinase activities) were purchased from Sigma and used without further purification. Sodium dihydrogen phosphate (NaH_2PO_4, BDH)) was used to prepare the buffer solutions. $NaBH_4$ (Aldrich) in NaOH (0.1 M BDH) was prepared fresh daily. HCl (BDH) and CH_3OH (EMD) were of analytical grade or better.

3.2.3 SAMPLES

The commercial product, kelp powder, was purchased from a local food store (Edmonton, Canada).

3.2.4 PROCEDURES

Figure 3.1 shows a schematic representation of the different procedures utilized for the extraction of arsenic species from kelp samples after enzyme hydrolysis.

3.2.4.1 Enzyme hydrolysis of seaweed samples at room temperature

Duplicate samples of kelp (0.1 ± 0.0001mg) were weighed in 50-mL centrifuge tubes and mixed with a solution containing the enzymes (50 mg) previously dissolved in phosphate buffer (10 mL, 0.1 M). Enzymes used were cellulase/hemicellulase (pH 5.3), cellulase/pectinase (pH 4.5), and lysing enzymes (pH 5.5). Equivalent amounts of enzymes were used for each treatment. The tubes containing the enzyme-sample mixture were capped and left for overnight incubation (~12 hours) at room temperature.

Control duplicate samples were prepared in a similar manner except that phosphate buffer was added instead of enzyme. Blank duplicate samples only contained enzymes and buffer.

3.2.4.2 Enzyme hydrolysis of seaweed samples over different incubation times

Triplicate samples of kelp were prepared as described in Section 3.2.4.1 except that only a mixture of cellulase/pectinase was used for enzyme hydrolysis. Samples were incubated at 37 °C in a thermostatic water bath for 8 or 24 hours.

Control and blank samples were prepared in a similar manner as described in Section 3.2.4.1

3.2.4.3 Extraction of samples and total digestion of residues

After incubation, each sample was extracted with methanol (10 mL), sonicated in an ultrasonic bath for 10 minutes and centrifuged for 20 minutes at 2500 rpm. The supernatant was decanted and placed in a 100-mL beaker. The extraction process with the aid of sonication was repeated one more time for each sample. The extracts were combined in the beaker, evaporated to near dryness and reconstituted to 20 mL with deionized water.

Residues from extraction were transferred to 100-mL beakers and combined with 10 mL of concentrated HNO_3/H_2SO_4 (3:1). Beakers were covered with watch glasses and residues were-soaked overnight at room temperature. Residues were then gently boiled for 5-6 hours, until the evolution of brown fumes ceased. Watch glasses from beakers were removed and residues were further heated for 1-2 hours until digested solutions were colorless or pale yellow. Final volume in beakers was 1-5 mL. After cooling to room temperature, digested solutions were neutralized, transferred to 50-mL centrifuge tubes and further diluted to 20 mL.

3.2.4.4 Microwave decomposition of extracts and digested residues

A 5-mL aliquot from each reconstituted extract or residue was placed in a 50-mL centrifuge tube and mixed with $K_2S_2O_8$ (1.0 g), NaOH (0.5 g) and

deionized water (5 mL). Sample tubes were loosely capped and placed in a microwave oven. The microwave oven was operated at full power setting (550W) for 5 minutes followed by a 5-minute cooling period. The heating-cooling cycle was repeated a further five times. Decomposed extracts and residues were then further diluted to 20 mL with deionized water.

3.2.4.5 FI-HG-AAS analysis

Decomposed extracts and residues were directly analyzed for arsenic by using FI-HG-AAS. Volume of injection was 500 µL and arsines generation took place as described above. Peak areas of AA signals were measured and standard arsenate solutions were used for calibration. Blank solutions were also prepared, treated in the same way as samples and standards, and used for signal correction.

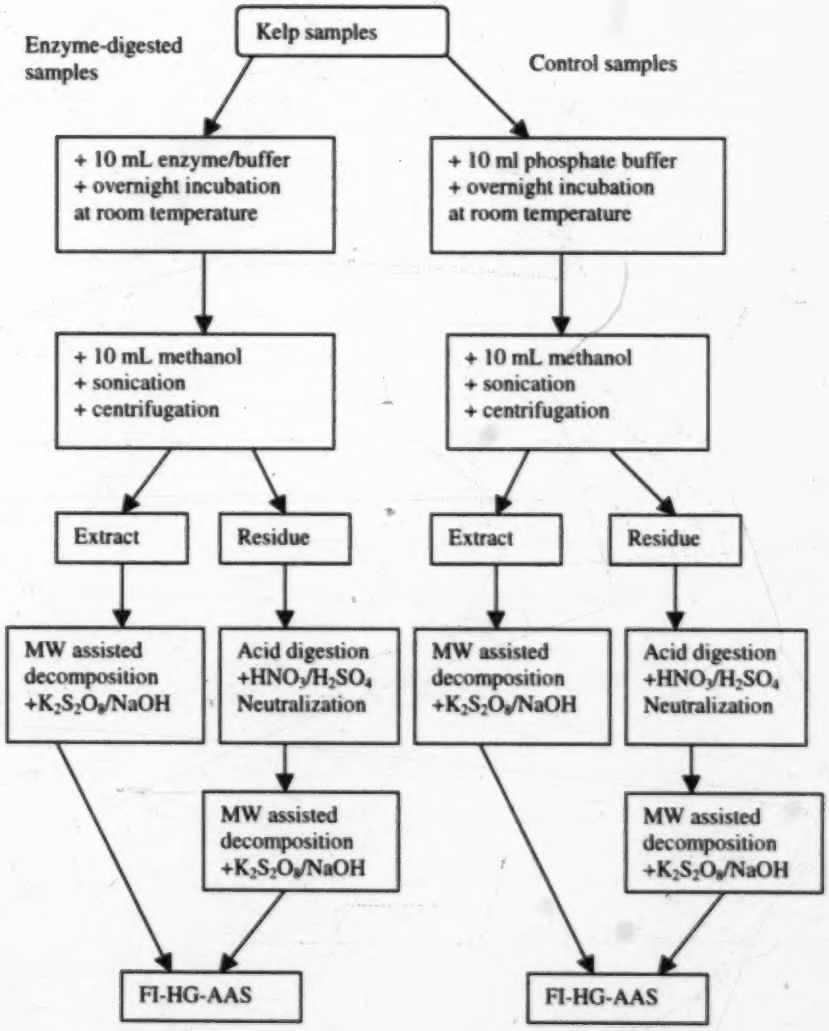


Figure 3.1: Flowchart for extraction procedures of arsenic species from kelp samples

3.4 RESULTS AND DISCUSSION

Arsenic concentrations in the samples were obtained from a calibration curve constructed with arsenate standards (Figure A4 in Appendix A, data shown in Table B7 in Appendix B). From a separate experiment, total arsenic in kelp samples was 25.0 ± 0.3 μg of arsenic per gram of dry sample (n=3). (data not shown, procedure under Section 3.2.4.3 except that samples were not previously extracted). Duplicate samples were prepared for each condition and three or four injections were made for the FI-HG-AAS analysis. The extraction efficiency for each sample was calculated as the ratio of the arsenic content in the extract, divided by the total content of arsenic in the sample. Figure 3.2 shows the percent arsenic in extracts and residues of kelp samples after enzyme hydrolysis at room temperature. Arsenic concentrations in extracts and residues are summarized in Table B8 in Appendix B.

The extraction efficiencies in the extracts after enzyme hydrolysis ranged from 65 to 68 % with standard deviations ranging from 2-5%. The extraction efficiency in the control extracts was 69 ± 2%. In the literature, extraction efficiencies have been reported in the range 81-85% of arsenic in methanol-water extracts from similar seaweed samples[54,60]. As mentioned previously (Section 2.3.3.1, Chapter 2) results are not directly comparable due to the different types of algae used for the preparation of the commercial product. In residues after enzymatic treatment, the arsenic content amounted to 24-25% with standard deviations ranging from 1-5%. Residues from control samples contained 29 ± 1% of arsenic.

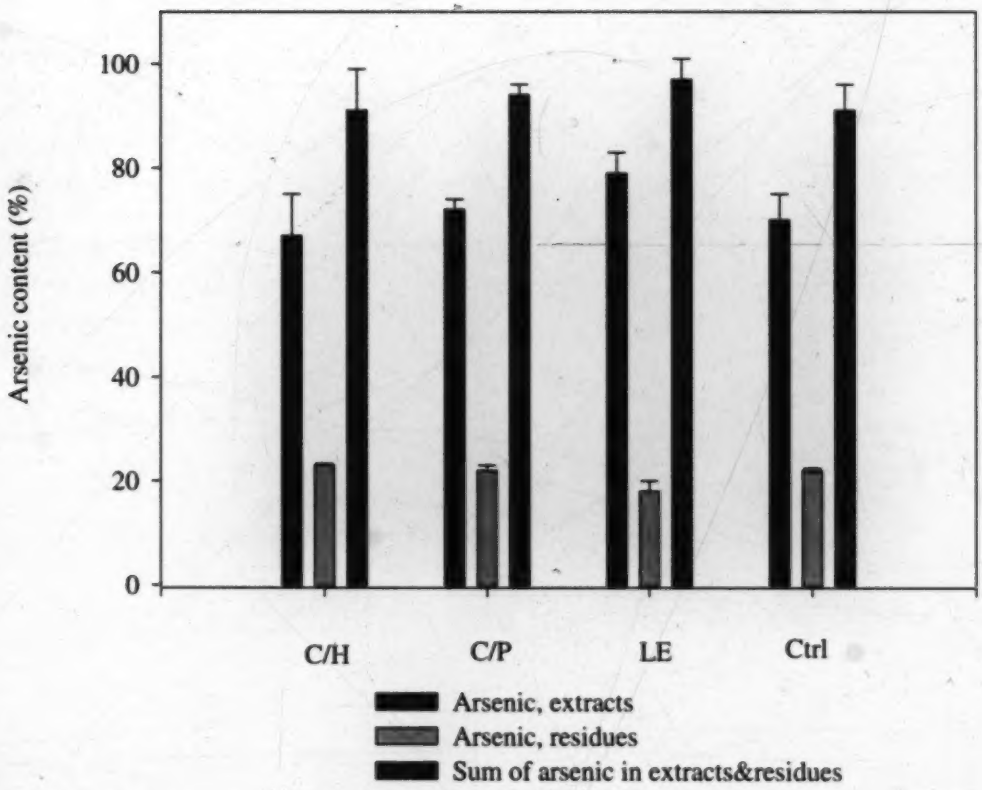


Figure 3.2: Percent arsenic in kelp samples (n=2) after enzyme hydrolysis with cellulase/hemicellulase (C/H), cellulase/pectinase (C/P) and lysing enzymes (LE) at room temperature for 12 hours. Control samples (Ctrl) did not contain enzyme. Kelp samples were prepared under Section 3.2.4.1 in Chapter 3 as shown in Figure 3.1

The mass balance of arsenic in the samples, the sum of the extracted arsenic and the residual arsenic, provides an alternative way to evaluate the performance of the extraction procedure. Ideally, this sum should be equal to the total amount of arsenic in the sample. In the samples after enzyme hydrolysis the mass balance of arsenic ranged from 89-93%, and was 98% in the control samples. Arsenic losses might be attributed to non-quantitative transfer of liquids fraction through the different steps of the procedure. In the case of the enzymatic-digested extracts, this problem becomes more serious as enzymes tend to adhere to the walls of glass and plastic containers. The analyte species in the extract might be trapped within the enzyme dispersion and thereby, might not be available for transfer.

As seen from results shown in Figure 3.2, there was not a significant difference in terms of arsenic extracted when comparing results from enzyme-digested and control extracts. This was also supported by the results of residual arsenic content.

When kelp samples were incubated with cellulase/pectinase at 37 °C for 8 and 24 hours, extractabilities of arsenic were not significantly different compared to the results from preceding experiment.

Typical FI-HG-AAS signal are shown in Figure 3.3. Arsenic concentrations in the samples were obtained from a calibration curve constructed with arsenate standards (Figure A5 in Appendix A, data shown in Table B9 in Appendix B). Arsenic concentrations in extracts and residues are summarized in Table B10, Appendix B.

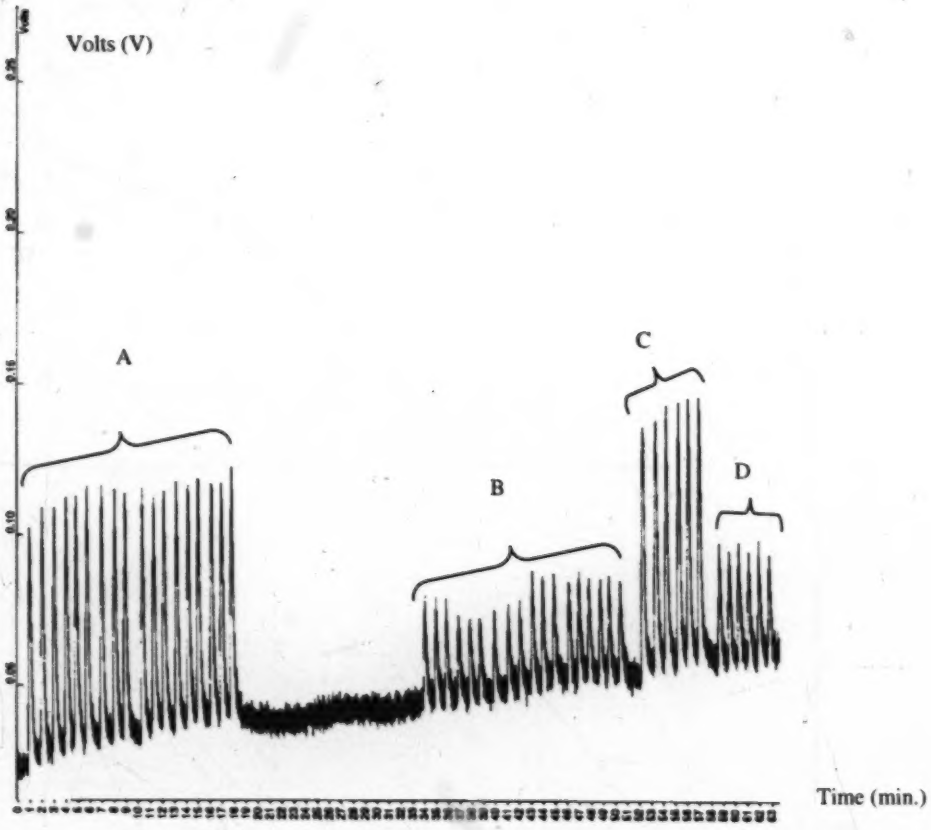


Figure 3.3: FI-HG-AAS signals of arsenic in kelp samples after enzyme hydrolysis with a mixture of cellulase/pectinase at 37°C for 8 and 24 hours (under procedure in Section 3.2.4.2 in this Chapter). Regions A and B show signals from digested extracts and residues, respectively. Regions C and D show signals from control extracts and residues. Both extracts and residues were microwave-digested with $K_2S_2O_8$/NaOH prior to FI-HG-AAS analysis

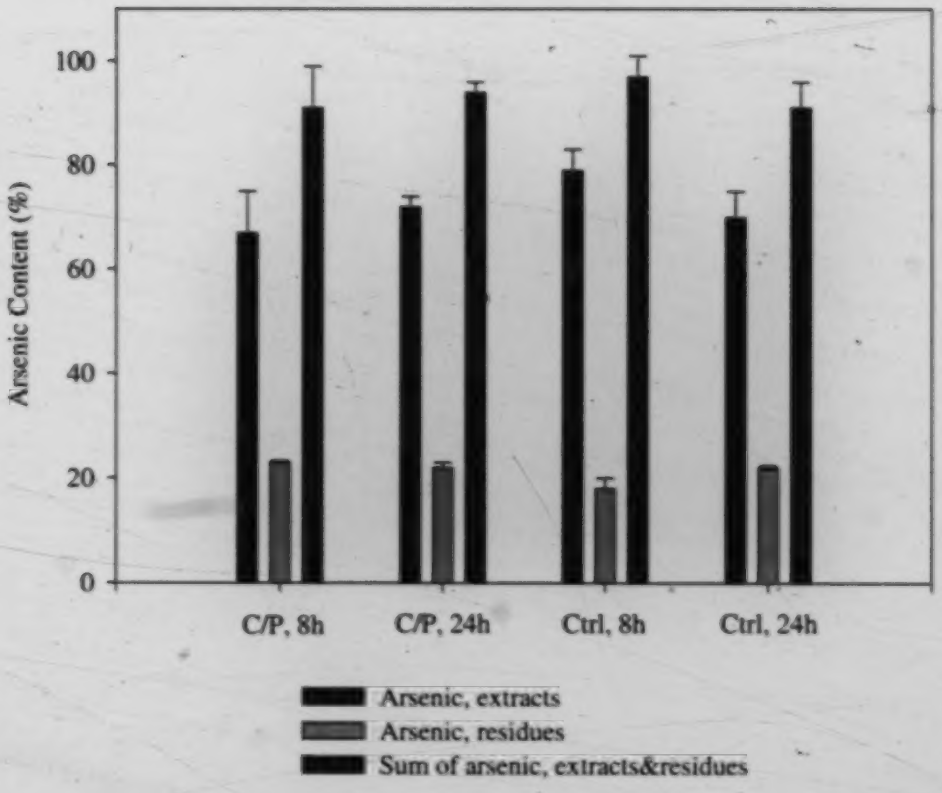


Figure 3.4: Percent arsenic in kelp samples (n=2) after enzyme hydrolysis with a mixture of cellulase/pectinase (C/P) at 37°C for 8 and 24 hours. Control samples (Ctrl) did not contain enzyme. Kelp samples were prepared under Section 3.2.4.2 in Chapter 3 as shown in Figure 3.1

Extraction efficiencies, shown in Figure 3.4, were calculated in the same manner as described in Section 3.4. Extraction efficiencies were 67 ± 8% and 72 ±2% in the extracts incubated with enzyme for 8 and 24 hours, respectively. In the control samples, extraction efficiencies were 70 ± 5% and 79 ± 4%. for 8 and 24 hours of incubation (no enzyme added). Correspondingly, arsenic content in residues amounted to 23 ± 0.3% and 22 ± 1% for enzyme-digested samples and 18 ± 2% and 22 ± 0.4%, respectively.

Mass balances of arsenic in enzyme-digested samples accounted for 91-94% of the total arsenic in kelp samples. In control samples, percentages ranged from 91-97%. The overall mass balance of arsenic in all samples accounted for more than 90% of the total arsenic content. Arsenic losses may then be explained by similar reasons as presented in previous paragraphs.

Although it was expected that both increased temperature and length of incubation would maximize the enzymatic activity[61], these factors did not seem to affect the extraction efficiencies as indicated by the non-significant differences in extractabilities of arsenic of digested and control samples. This might be due to the use of non-optimized conditions (temperature and time of incubation) for each enzyme preparation.

In general, differences in hydrolytic activity among the enzyme preparations tested were not significant under the present experimental conditions. Even though the structural model of cell walls in algae is still controversial, it is commonly accepted the presence of polysaccharides such as cellulose, hemicellulose and pectic materials as part of the cell wall skeleton.

Enzymes were then chosen according to this general composition of cell walls. However, extraction efficiencies of arsenic were not increased nor were visual changes noticed on the sample texture after enzyme treatment. It seemed likely that enzymes were already inactive or inactivation might have been induced at some early stage in the procedure. Enzyme activities should be determined by standard assays for future experiments.

From these results, it is apparent that the enzyme preparations under study were not capable of digesting the seaweed samples. Given the chemical and structural complexity of the cell wall matrix in algae, it is then reasonable to think of the cell wall hydrolysis as a process resulting from the cooperative activity of large numbers of polysaccharide hydrolases with complementary specificities[59]. For instance, according to the manufacturer, the commercial cellulase used in the experiment contains only an endo-β-1, 4, glucanase. However, the efficient conversion of cellulose to glucose requires the concerted action of several enzymes, such as, endo-β-1, 4, glucanase, cellobiohydrolase and β-glucosidase[59]. Results presented in the following paragraphs seemed to support this conclusion.

Duplicate samples of mussels, mushroom and tobacco leaves were incubated with pancreatin, lysing enzymes and cellulose/pectinase, respectively (room temperature, 12 hours), following the same protocol described in Section 3.2.4. Enzymes were chosen according to the general composition of each matrix. Arsenic concentrations in extracts and samples after total digestion are shown in Table B11 in Appendix B.

The extraction efficiencies of arsenic from mussels, mushroom and tobacco leaves after enzyme hydrolysis were 90, 67, and 97%, respectively. In the case of mussels and mushroom, extraction efficiencies were similar to the values reported by using solvent extraction (mussels[62]: 84-99%; mushroom[63]: 80%). No published data regarding arsenic extractability from tobacco leaves were available.

Essentially, animal tissues, such as mussels, are made up of proteins. After homogenization, the proteins may be more exposed to enzymatic action. Also, pancreatin contains many enzymes, mainly proteases (trypsin, pepsin) and some amylase, lipase and ribonuclease activities. The combination of different enzymes, sample homogenization and the lack of cell walls may have contributed to the efficient digestion of the homogenized tissue and increased extraction efficiency of arsenic.

In the case of tobacco leaves, the enzymatic treatment with cellulase/pectinase helped solubilize most of the arsenic present in the sample. This enzyme preparation seemed to be more suitable for degradation of plant cell wall. Also, this sample was in the form of a finely divided powder. Similarly, homogenized mushroom samples were treated with lysing enzymes, a commercial preparation containing celullase, protease, and chitinase activities Chitin (a β-1,4-linked polymer of *N*-acetylglukosamine) together with β-1,3-glucans) are the major components of fungal cell walls[64]. Total arsenic concentrations in the present mushroom samples were low, in agreement with the general trend for arsenic concentrations in terrestrial plants[63]. Arsenic extractability was 67% while 80% was reported for a large variety of edible mushroom samples[63].

Results were not strictly comparable because of the difference in samples analyzed.

In summary, these results seemed to indicate that enzymatic hydrolysis of plant and animal tissue requires a multi-enzyme preparation involving targeted specificity towards both the type of sugar and the linkage in the sample under study, in agreement with Hazlewood and co-workers[59]. Therefore, multi-enzyme preparations rather than individual enzymes should be screened for cell wall-degrading activities and their enzyme activity quantified by standard assays. Also, sample homogenization seemed to influence the performance of the enzymatic action, as reported by Surnakki and co-workers in the enzymatic bleaching of chemically modified wood pulp[65].

Toward the end of this study, some of the kelp extracts were analyzed by HPLC-ICP-MS (as described in Section 2.2.4 in Chapter 2). Figure 3.5 and 3.6 show the profiles of the control extracts (no enzyme added) and the enzyme-digested extracts, respectively. The HPLC profiles were essentially identical. No changes in retention times or relative intensity of peaks were noticed. This confirmed the enzymatic treatment did not change the native arsenic species present in kelp.

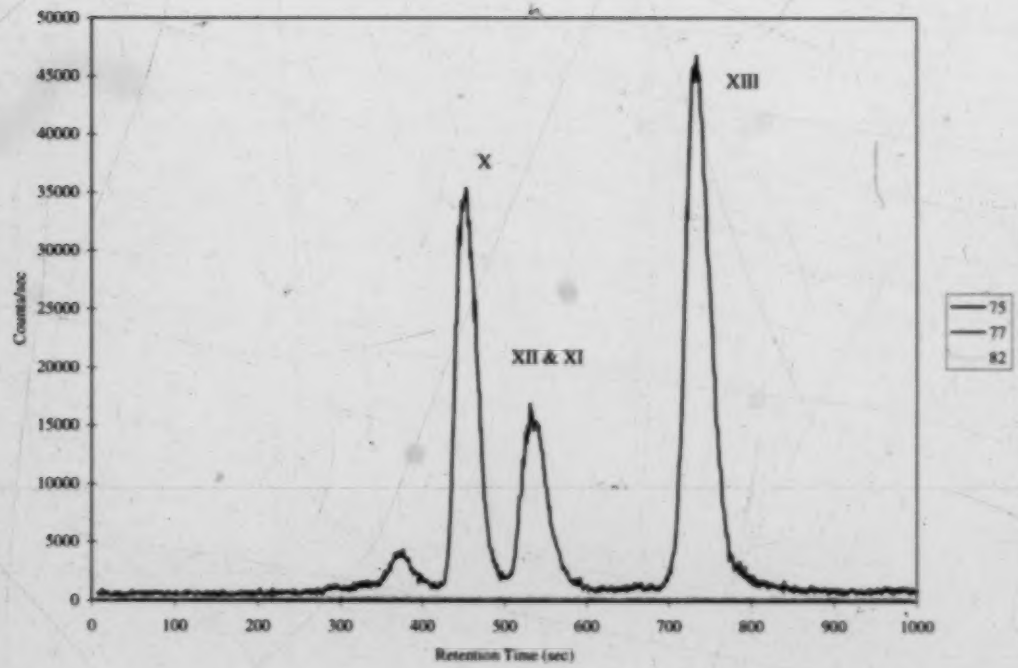


Figure 3.5: Chromatogram obtained from HPLC-ICP-MS analysis of a control kelp extract (no enzyme added, prepared as shown in Figure 3.1). HPLC conditions were as in Figure 2.3-5 in Section 2.3.2, Chapter 2. Peaks X-XIII corresponded to arsenosugars (structures shown in Figure 1.1 in Section 1.1, Chapter 1)

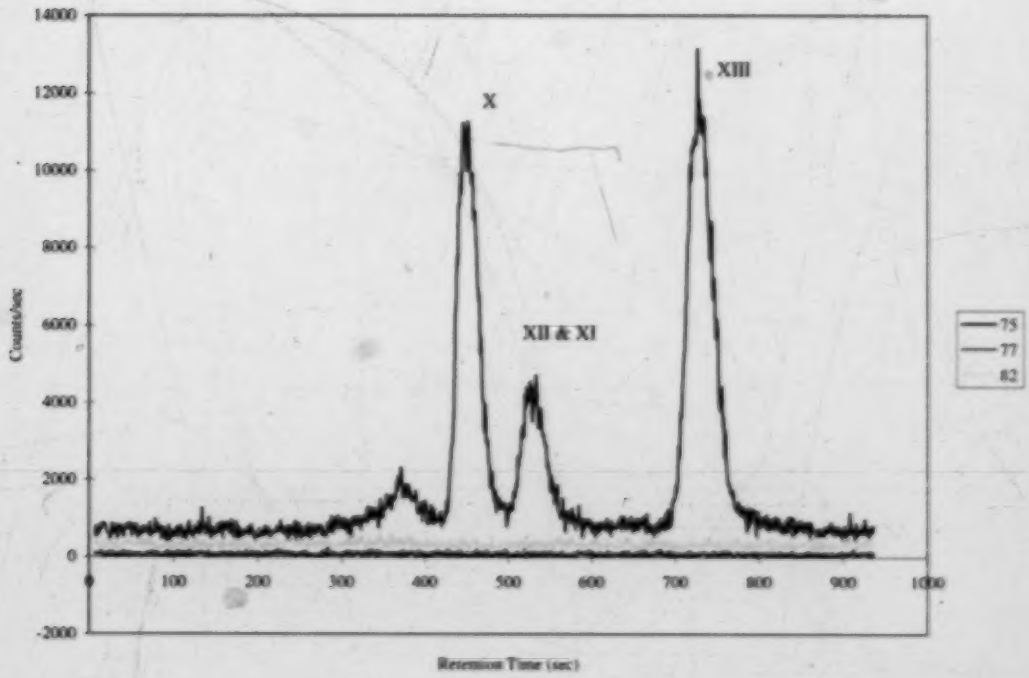


Figure 3.6: Chromatogram obtained from HPLC-ICP-MS analysis of a kelp extract (enzyme-digested with cellulase/pectinase at 37°C for 8 h, prepared as shown in Figure 3.1). HPLC conditions were as in Figure 2.3-5 in Section 2.3.2, Chapter 2. Peaks X-XIII corresponded to arsenosugars (structures shown in Figure 1.1 in Section 1.1, Chapter 1)

CHAPTER 4

SEQUENTIAL EXTRACTION OF ARSENIC SPECIES FROM SEAWEED SAMPLES USING BATH AND PROBE SONICATION

4.1 INTRODUCTION

Results from the previous chapter showed that enzyme hydrolysis on seaweed samples did not improve the extraction efficiencies of the arsenic speciesin the seaweed sample analyzed. As previously discussed, the use of a multi-enzyme preparation is strongly recommended given the structural complexity of the samples under study.

However, the structural diversity of the samples in which arsenic can be found naturally poses more challenges on developing extraction procedures for elemental speciation. For instance, enzymes were chosen according to the sample composition. In some cases, however, structure and composition are still issues not resolved. As a result, targeting specific enzymatic action may be difficult or even non-practical. Furthermore, the use of specific enzyme(s) for specific sample(s) in routine speciation analysis may not be feasible in terms of economy (enzymes are rather expensive products).

An alternative to the use of enzymes for tissue homogenization might be sonicating samples with an ultrasonic probe. Cavitation produced by ultrasound

energy causes disruption of cell walls in cells, tissues, bacteria and yeast[30] with concomitant release of their contents in the surrounding liquid. In this context, acoustic cavitation and the consequent disruptive action have resulted in high extractive power in solid samples subjected to ultrasound treatment, as reviewed in Section 1.3.

Therefore, the objective of this study was to investigate sonication to disrupt the cell walls in algae, and consequently improve the extractabilities of arsenic species from seaweed samples. Sonication was performed in combination with solvent extraction and enzyme hydrolysis.

4.2 EXPERIMENTAL

4.2.1 INSTRUMENTATION

An atomic absorption spectrophotometer (Varian, SpectrAA-5) was used for detection of arsenic. The FI-HG-AAS system has been described in Section 2.2.1. An ultrasonic probe (Vibracell, Sonics and Materials) with adjustable power control (0-100% in arbitrary units) and an ultrasonic bath (Sonicor) with constant power input were used for the sonication experiments.

4.2.2 STANDARDS AND REAGENTS

Deionized water from a Milli-Q ultrapure water system (Millipore) was used for the preparation and dilution of all reagents, samples, and calibration standards as described in Section 2.2.3. Na_2HAsO_4 (Sigma) was used to prepare arsenic calibration standards.

Cellulose (C-9422) and hemicellulase (H-7649) were purchased from Sigma and used without further purification. NaH_2PO_4, (BDH) was used to prepare the buffer solutions. $NaBH_4$ (Aldrich) in NaOH (0.1 M BDH) was prepared fresh daily. HCl (BDH) and CH_3OH (EM) were of analytical grade or better.

4.2.3 SAMPLES

The commercial product, kelp was purchased from a local food store

(Edmonton, Canada). Kelp was in the form of a finely divided powder. The product was kept in its original package away from direct sunlight.

4.2.4 PROCEDURES

4.2.4.1 Sequential extraction of arsenic species from kelp samples with bath and probe sonication

Triplicate samples of kelp (0.1 ± 0.0001mg) were weighed, placed in 50-mL centrifuge tubes and mixed with 10 mL of methanol-water (1:1, v/v). Each sample was then sonicated in an ultrasonic bath for 20 minutes. After centrifugation (20 minutes, 2500 rpm) each supernatant was removed and placed in a 100-mL beaker. The extraction process with the aid of sonication was repeated one more time for each sample. Extracts were combined in beakers, evaporated to near dryness and reconstituted to 20 mL with deionized water. Blanks were prepared in the same manner, except that no sample was added.

Residues from previous step were mixed with portions of 10 mL of methanol/water (1:1) and sonicated with the probe at 70% ultrasound amplitude, (arbitrary units) for 5 minutes. Sonicated samples were then centrifuged (2500 rpm, 20 minutes) and supernatants were decanted and placed in separate beakers. Samples were extracted one more time with the aid of ultrasound energy. Supernatants were combined in beakers, evaporated, and reconstituted to 20 mL with deionized water. This procedure is summarized in Figure 4.1.

4.2.4.2 Sequential extraction of kelp samples with bath and probe sonication followed by enzyme hydrolysis

Triplicate samples of kelp (100 mg) were prepared as in Section 4.2.4.1, except that residues were incubated with cellulase/hemicellulase (10 mg each in 10 mL phosphate buffer, pH 5.0) at 37 °C for 24 hours. After incubation, residues were further extracted as in Section 4.2.4.1 except that only bath sonication was used. A flowchart of the procedure is shown in Figure 4.2.

Procedures for total digestion of residues, microwave decomposition of extracts and digested residues and FI-HG-AAS analysis were the same as in Sections 3.2.4.3-3.2.4.5 in Chapter 3.

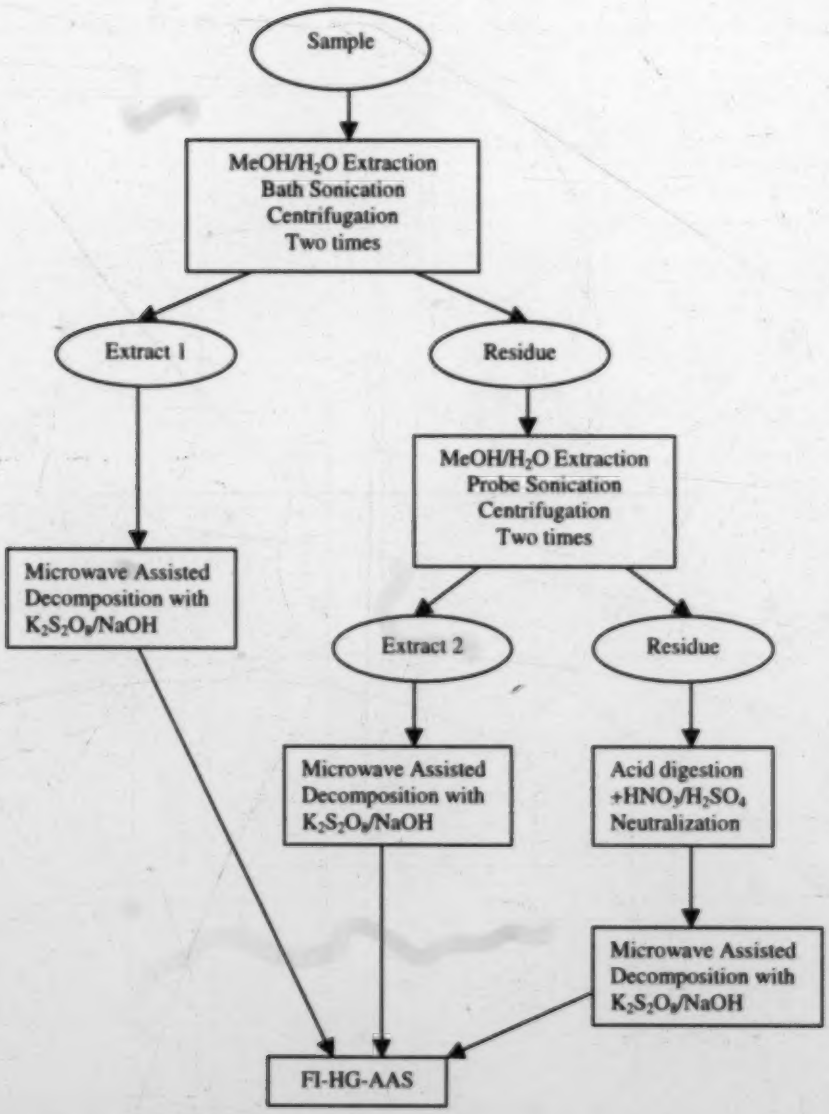


Figure 4.1: Flowchart for sequential extraction of kelp samples (bath + probe sonication)

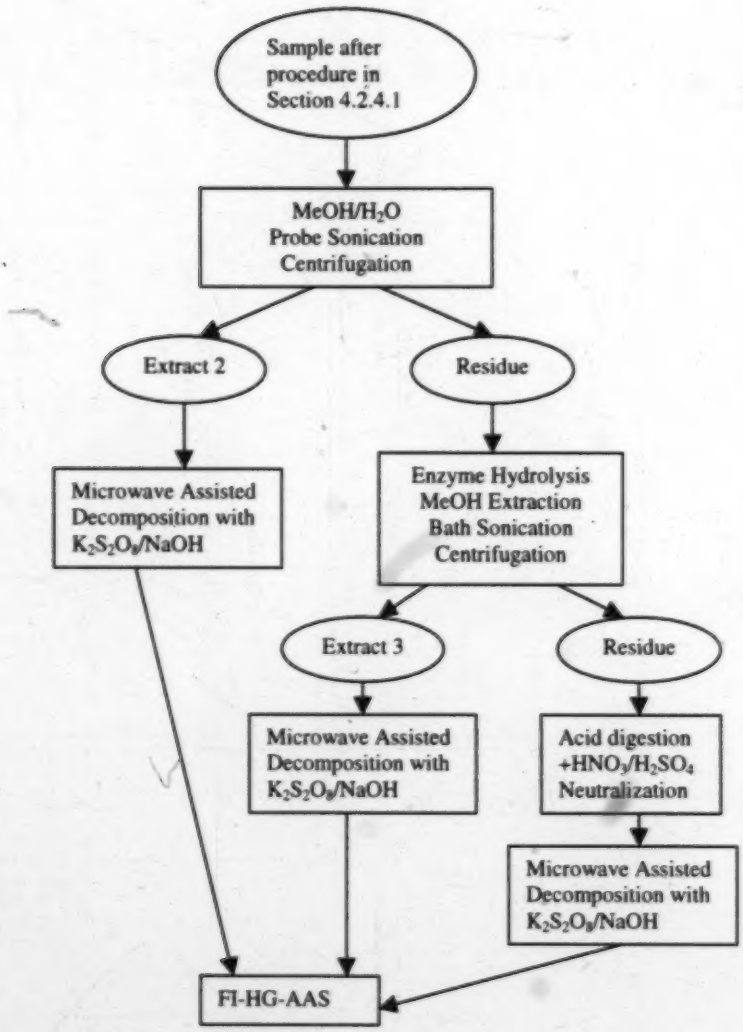


Figure 4.2: Flowchart for sequential extraction of kelp samples (bath + probe sonication) followed by enzyme hydrolysis

4.3 RESULTS AND DISCUSSION

Figure 4.3 shows typical FI-HG-AAS signals of arsenic and Figure 4.4 shows the percent arsenic in extracts and residues of kelp samples (n=3) prepared under Section 4.2.4.1. Extraction efficiencies were calculated as in Section 3.4 in Chapter 3.

Arsenic concentrations in the samples were obtained from a calibration curve constructed with arsenate standards (Figure A6 in Appendix A, data in Table B12 in Appendix B). Arsenic concentrations in extracts and residues are summarized in Table B13 in Appendix B. From a separate experiment, total arsenic in kelp samples was 24 ±1 μg of arsenic per gram of dry sample (n=5), (data not shown, procedure under Section 3.2.4.3 except that samples were not previously extracted).

In this first experiment, 83 ± 7% of arsenic was present in the water-methanol phase after bath sonication, in agreement with values reported in fresh brown algae[60] (81%) and the commercial product[54] (85%). Probe sonication with methanol/water yielded a further 11 ± 3 % of arsenic in the extracts. Lastly, arsenic content in the residues was 5.9 ± 1.3%.

The combined extraction efficiency after sequential bath and probe sonication amounted to 93%, however, standard deviations reported for the arsenic content in extracts and residues were also high, a situation which might be due to the heterogeneity of the sample, as reported by others[56].

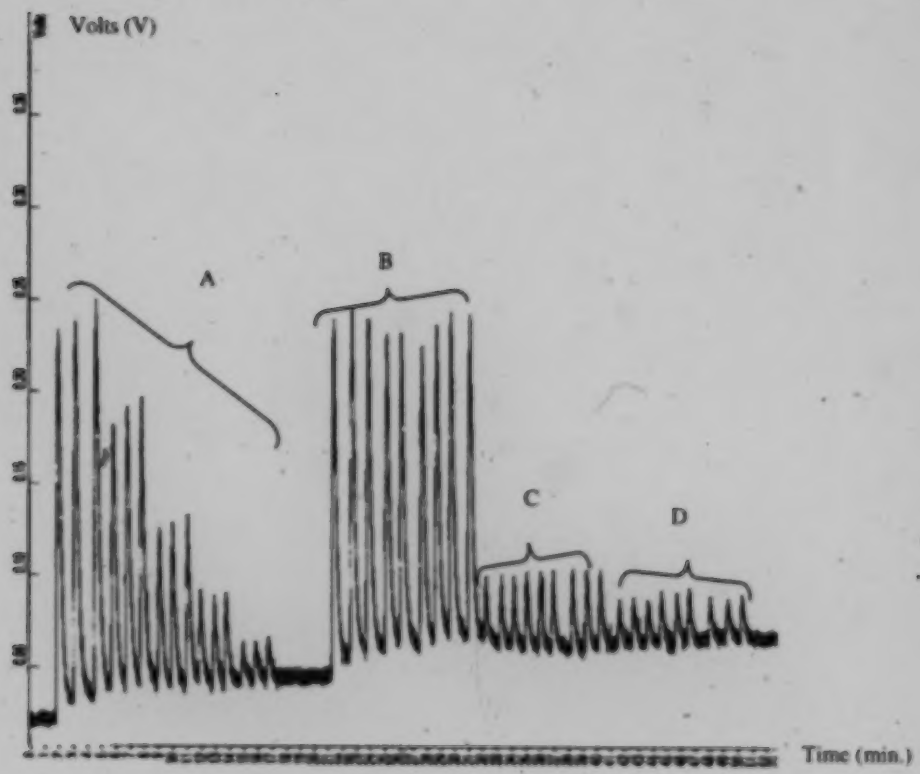


Figure 4.3: FI-HG-AAS signals of arsenic in kelp samples after sequential extraction with bath and probe sonication (under procedure in Figure 4.1). Region A shows signals from As^{V} standards. Regions B shows arsenic signals from extracts after bath sonication. Residues were subsequently extracted with probe sonication (Region C). Region D shows arsenic signals from residues after procedures in B and C. Both extracts and residues were microwave-digested with $K_2S_2O_8$/NaOH prior to FI-HG-AAS analysis

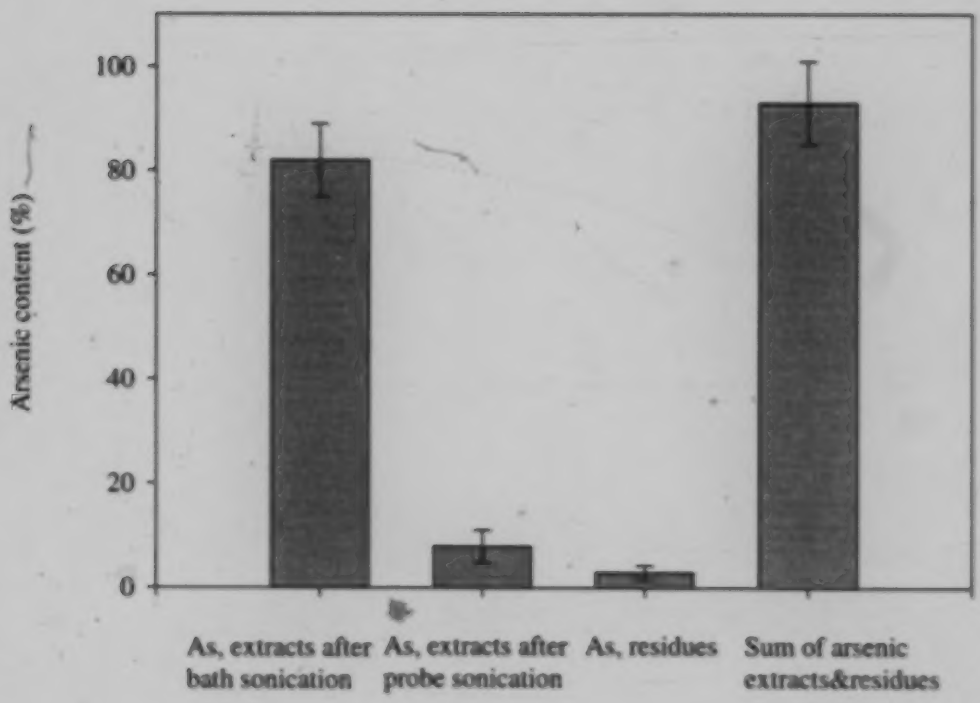


Figure 4.4. Percent arsenic in kelp samples (n=3) after sequential extraction with bath and probe sonication. Percent arsenic was calculated as described in Section 3.4 in Chapter 3. Kelp samples were treated under Section 4.2.4.1, Chapter 4 as shown in Figure 4.1

Considering that about 80% of the arsenic present in kelp samples is commonly extracted by the usual procedure (methanol/water extraction plus bath sonication), the improvement in the extraction efficiency of arsenic with the present procedure was not dramatic. Even though the ultrasonic probe is claimed to be the best source of high power ultrasound[45], results did not seem to indicate extensive cell walls disruption. The samples seemed to keep their initial powdery appearance.

On the other hand, combined extraction efficiencies of arsenic from kelp samples (arsenic from bath sonication + arsenic from probe sonication) are within reasonable agreement with those reported in the literature[54,60]. The mass balance for arsenic in the kelp samples ranged from 96-104%, which is also in agreement with that already reported (Section 3.4 in Chapter 3) in extracts not enzyme-digested.

The limit of extraction efficiency was in the range 85-90% and neither enzyme hydrolysis nor probe sonication proved useful to release the remaining 10-15% of arsenic in the sample. This residual arsenic might well be complexed with the cell wall carbohydrates or trapped within the cellulose microfibrills, which form the skeleton of the cell wall[57].

A second experiment was designed to investigate a combined approach involving sequential extraction with bath and probe sonication followed by enzyme hydrolysis. Arsenic concentrations in extracts and residues were obtained from a calibration curve constructed with arsenate standards (Figure A7 in

Appendix A, data in Table B14 in Appendix B). Arsenic concentrations in extracts and residues are summarized in Table B15 in the Appendix B.

From a separate experiment, total arsenic in kelp samples was 20 ± 2 µg arsenic/g dry sample (n=3) (data not shown, procedure under Section 3.2.4.3 except that samples were not previously extracted). Extraction efficiencies were calculated as in Section 3.4, Chapter 3. Figure 4.5 shows the percent arsenic in extracts and residues of kelp samples.

The extraction efficiencies of arsenic were 58±5%, 27±2 % and 6.4±0.6% in extracts after sequential bath sonication, probe sonication and enzyme-hydrolysis, respectively. The residual arsenic was 8.8. ± 0.4%. The combined extraction efficiency of the extracting sessions was 91 ± 5%, which is not significantly different from results reported in preceding experiments in this Section. Apparently, a minor fraction of arsenic was additionally extracted after enzyme hydrolysis, however, it remains unclear, whether this effect was associated to some degree of cell wall degradation, or, if it was essentially due to the extraction/sonication step.

The sequential extraction procedure (described in Section 4.2.4.1) was also applied to two other commercial algae-based products, Yakinori and Nostoc, from red and terrestrial algae. Arsenic concentrations in extracts and total digested samples are summarized in Table 16 in Appendix B. Extraction efficiencies of arsenic in Nostoc and Yakinori extracts (n=2) were 11 ± 0.3% and 63 ±1.4%, respectively. Arsenic in residues amounted to 98 ± 0.2% and 21 ± 0.3% in Nostoc and Yakinori samples, respectively.

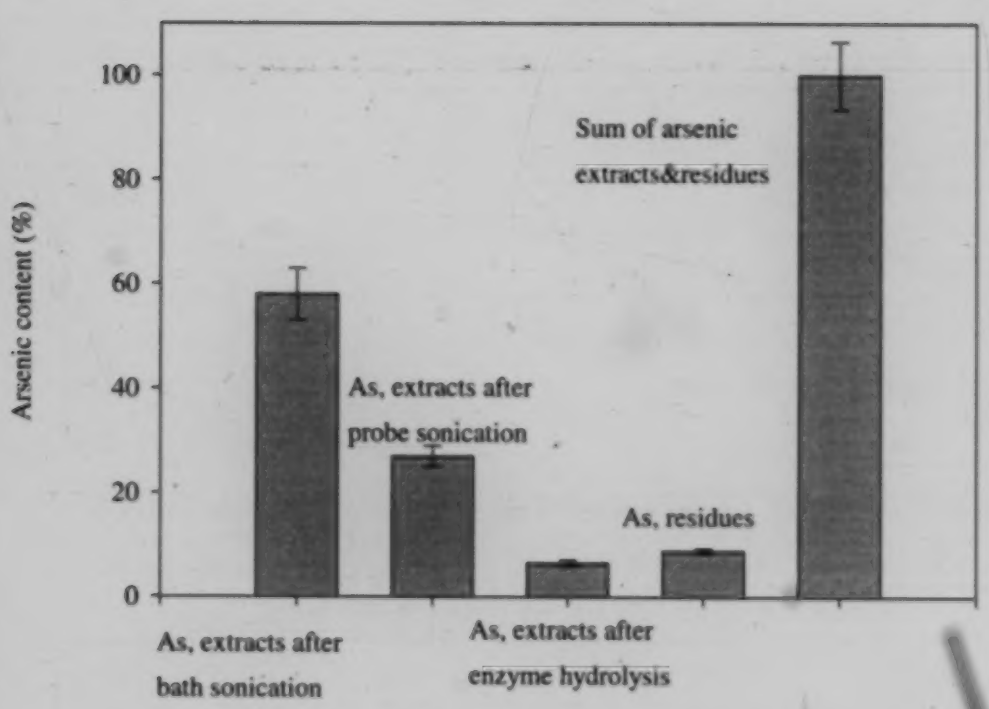


Figure 4.5: Percent arsenic in kelp samples (n=3) after sequential extraction with bath and probe sonication followed by enzyme hydrolysis. Percent arsenic was calculated as in Section 3.4 in Chapter 3. Kelp samples were incubated with cellulase/hemicellulase at 37 °C for 24 h under procedure in Section 4.2.4.2 in Chapter 4 as shown in Figure 4.2

In the literature, extraction efficiencies of arsenic have been reported to be in the range 64-76% and 34% from Yakinori[50] and Nostoc[54] samples respectively.

Although samples were treated as in Section 4.2.4.1, arsenic was only detected in the extracts after bath sonication. As Yakinori were in the form of thin baked sheets and Noctoc had a sponge-like appearance, samples were homogenized in a coffee grinder. However they were not converted into grain-sized particles. This might explain the poor arsenic extractabilities from non-powdered samples, particularly in the case of Nostoc. Finely divided solids are more homogenous as well as more easily dissolved and extracted because of their large surface area to volume ratio[30].

On the other hand, HPLC-ICP-MS profiles of these extracts showed that the sonication treatment did not further decompose arsenosugars to hydride-forming species, as presented in Figures 4.6-9.

Figure 4.6 and 4.7 show the chromatogram (HPLC ICP-MS analysis under section 2.3.2 in Chapter 2) of a Yakinori extract after bath sonication and probe sonication, respectively. Both profiles were essentially the same and no changes in retention times or relative intensity of peaks were observed. A similar result was found when Nostoc extracts were analyzed by HPLC-ICP-MS (Figure 4.8 and 4.9).

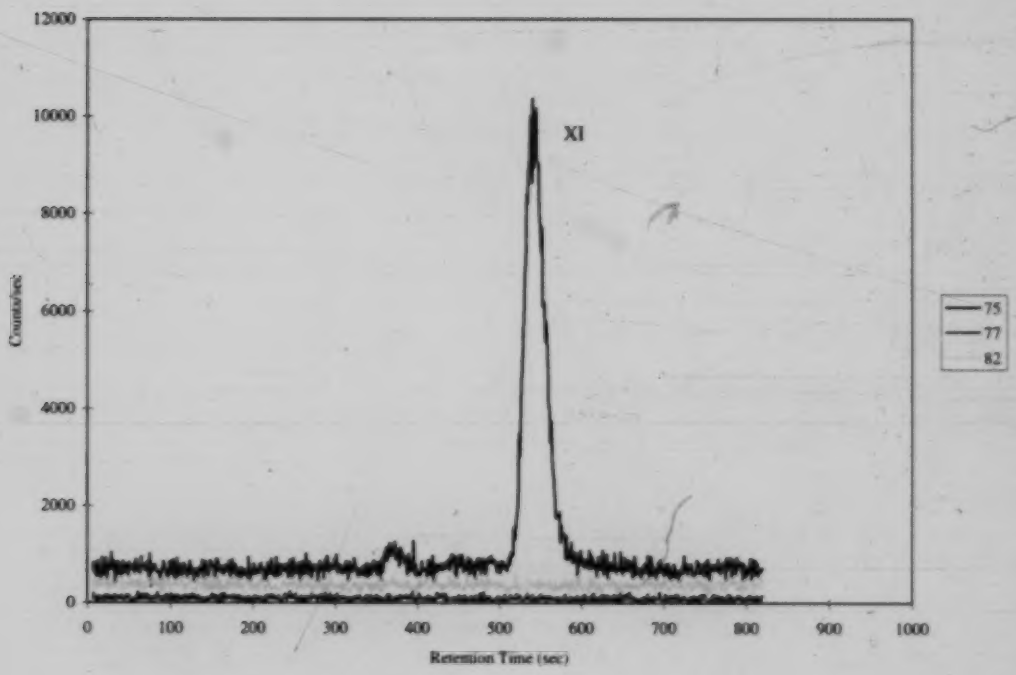


Figure 4.6: Chromatogram obtained from HPLC-ICP-MS analysis of a Yakinori extract after bath sonication (procedure in Section 4.2.4.1 as shown in Figure 4.1). HPLC conditions were as in Figures 2.3-5 in Chapter 2. Peak XI is an arsenosugar (structure shown in Figure 1.1, Chapter 1)

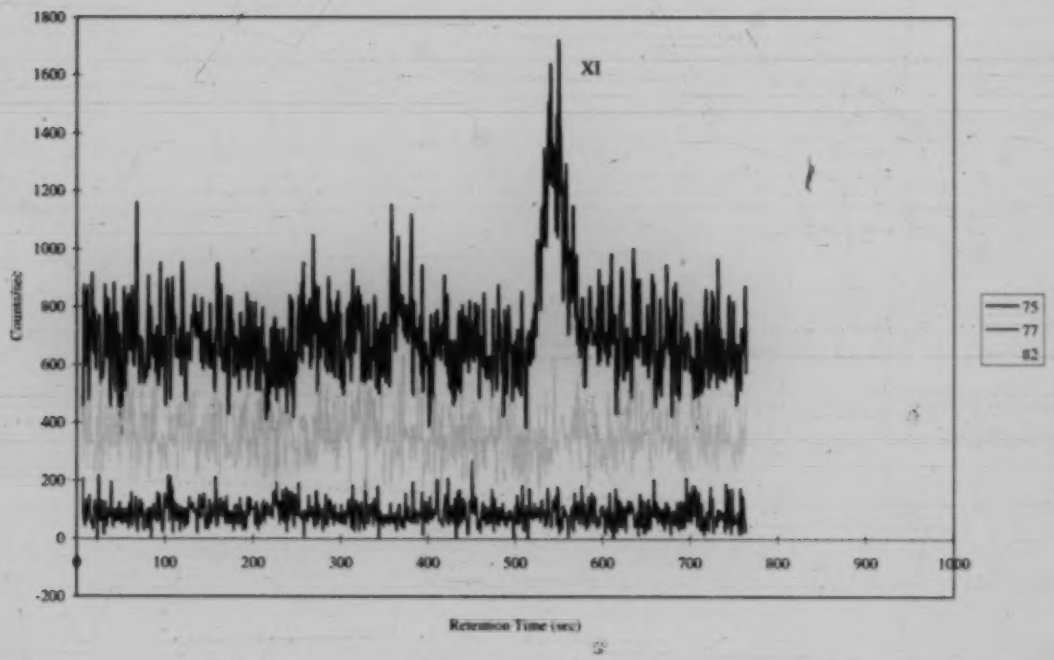


Figure 4.7: Chromatogram obtained from HPLC-ICP-MS analysis of a Yakinori extract after sequential extraction of the residue from Figure 4.6 using probe sonication (procedure in Section 4.2.4.1 as shown in Figure 4.1). HPLC conditions were as in Figures 2.3-5 in Chapter 2. Peak XI is an arsenosugar (structure shown in Figure 1.1, Chapter 1)

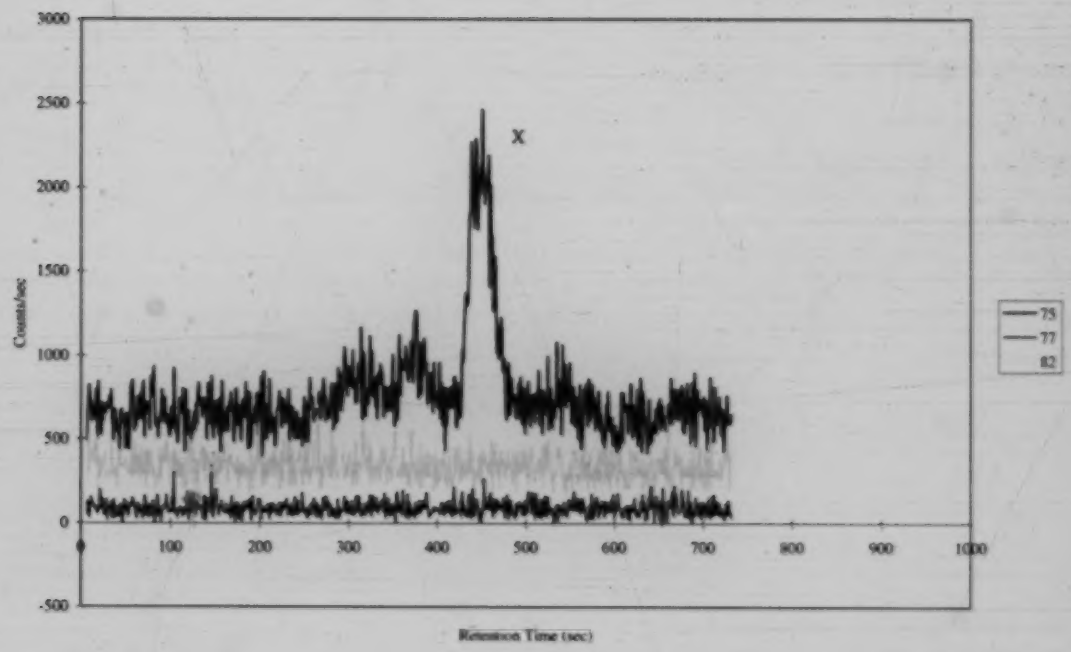


Figure 4.8 Chromatogram obtained from HPLC-ICP-MS analysis of a Noctoc extract after bath sonication (procedure in Section 4.2.4.1 as shown in Figure 4.1). HPLC conditions were as in Figures 2.3-5 in Chapter 2. Peak X is an arsenosugar (structure shown in Figure 1.1, Chapter 1)

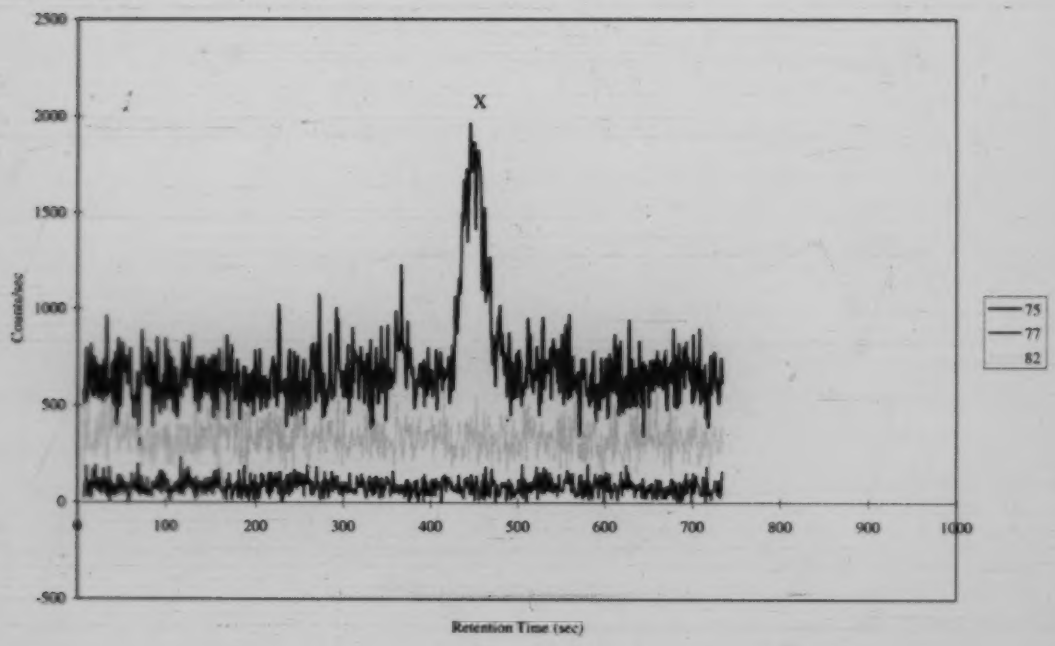


Figure 4.9 Chromatogram obtained from HPLC-ICP-MS analysis of a Noctoc extract after sequential extraction of the residue from Figure 4.8 using probe sonication (procedure in Section 4.2.4.1 as shown in Figure 4.1). HPLC conditions were as in Figures 2.3-5 in Chapter 2. Peak X is an arsenosugar (structure shown in Figure 1.1, Chapter 1)

In summary, the sequential extraction approach using sequential bath/probe sonication proved useful for the extraction of arsenic species from kelp samples. Extraction efficiencies of arsenic were close to the values reported by using the common extraction technique (methanol-water extraction with bath sonication). From HPLC-ICP-MS results it was shown that the sonication treatment did not decompose the native arsenic species in the samples analyzed. Also, the effectiveness of this treatment seemed to be influenced by the degree of sample homogenization.

CHAPTER 5

CONCLUSIONS

5.1 SUMMARY

Arsenosugars present in seaweed samples were not decomposed to hydride-forming species by cooking, diluted hydrochloric acid digestion or simulated gastrointestinal digestion under the experimental conditions employed.

Enzyme-assisted extraction *per se* did not improve the extraction efficiencies of arsenic under the present experimental conditions, particularly in the case of seaweed samples. When combined with sequential extraction using bath and probe sonication, extraction efficiencies (85-90%) were within reasonable agreement with values reported in the literature (80-90%). None of the treatments decomposed the native arsenic species.

5.2 EVALUATION

Although extraction efficiencies by the ultrasonic treatment were not improved with respect to the common solvent extraction technique, the procedure shows promise for better results. Homogenization of the sample may be a critical factor in obtaining high extraction efficiencies by the sequential extraction approach and may provide some room for improvement as reported by others[66].

In the case of enzyme-assisted extraction, the use of multi-enzyme preparations should be further investigated. Dissolution of cell wall polysaccharides in homogenized plant samples has been reported by others[40,57] using commercial enzymatic preparations. Also, the use of cell wall-degrading microorganism should be explored, particularly in the case of algal and fungal samples.

5.3 FUTURE DIRECTIONS

Results from this thesis indicated that arsenosugars metabolism is unlikely due to simple decomposition by cooking or gastrointestinal digestion prior ingestion of arsenosugars-containing seafood. In this context, *in vitro* microbial/enzymatic decomposition of arsenosugars, conducted in both anaerobic and aerobic conditions, should be studied in order to determine the actual cause of their metabolism in humans.

Incorporating sonication and enzymatic treatment within a sequential extraction approach proved useful for the extraction of arsenic from solid samples (seaweed) and did not change the native arsenic species. The sequential extraction approach should also be extended to include several other techniques such as microwave-assisted extraction or accelerated-solvent extraction, opening new possibilities for developing species extraction procedures.

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APPENDICES

This section contains:

Appendix A: Figures for finding arsenic concentrations

Appendix B: Data tables for figures in thesis and for Appendix A

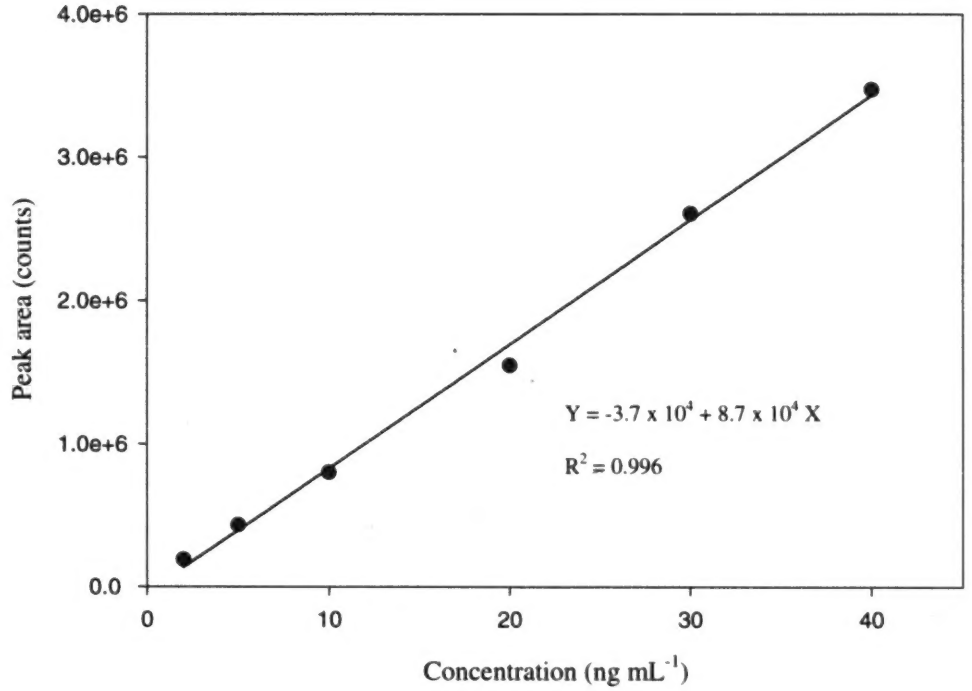

Figure A1: Calibration curve of arsenic standard solutions. Data presented in Table B1 in the Appendix B.

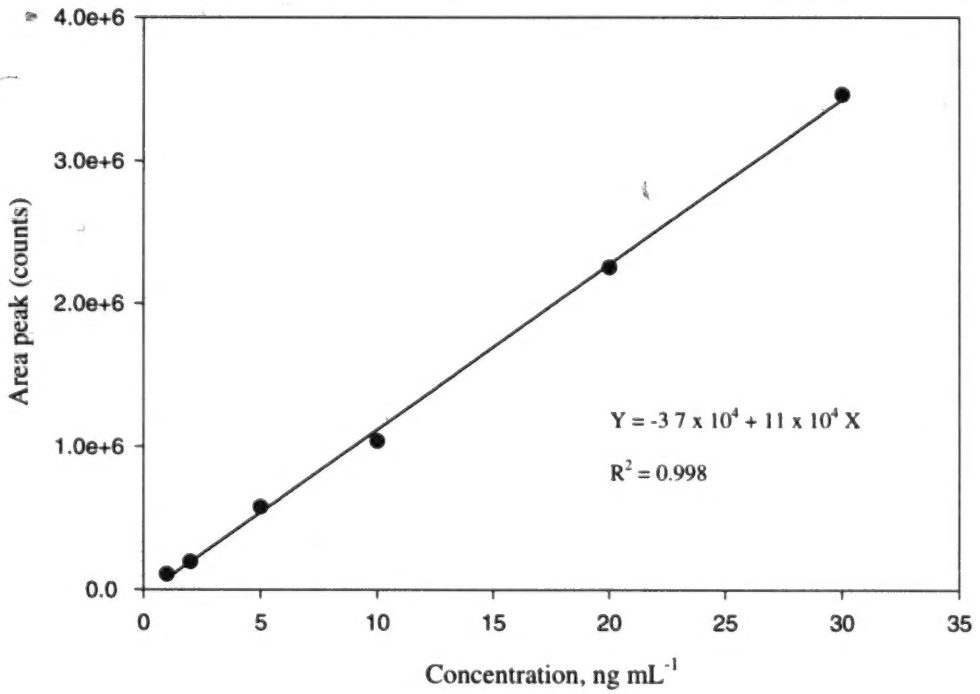

Figure A2: Calibration curve of arsenate standard solutions. Data presented in Table B3 in the Appendix B.

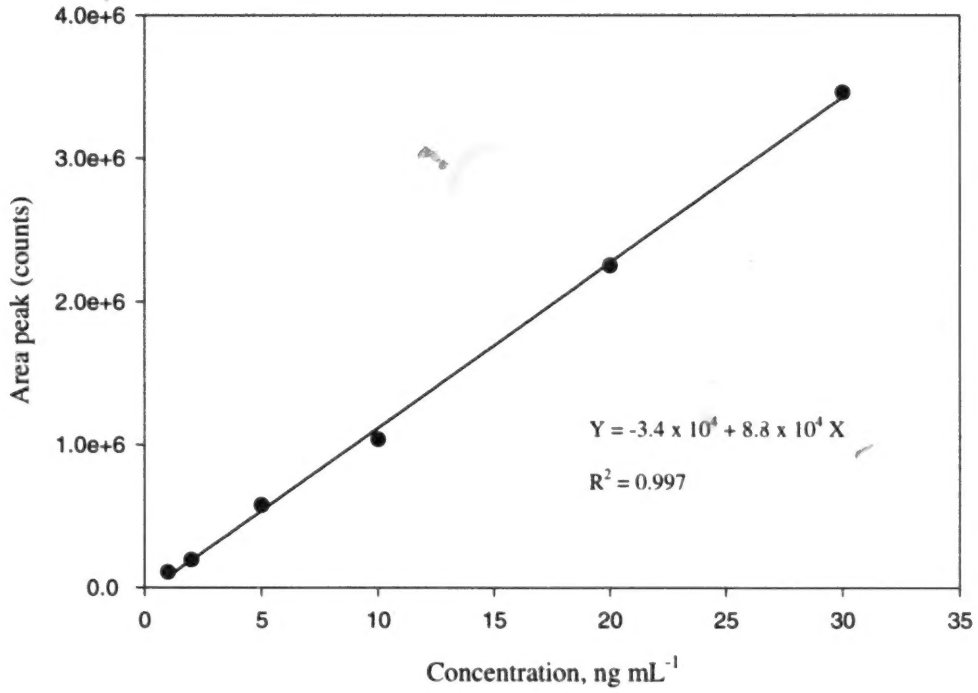

Figure A3: Calibration curve of arsenate standard solutions. Data presented in Table B5 in the Appendix B.

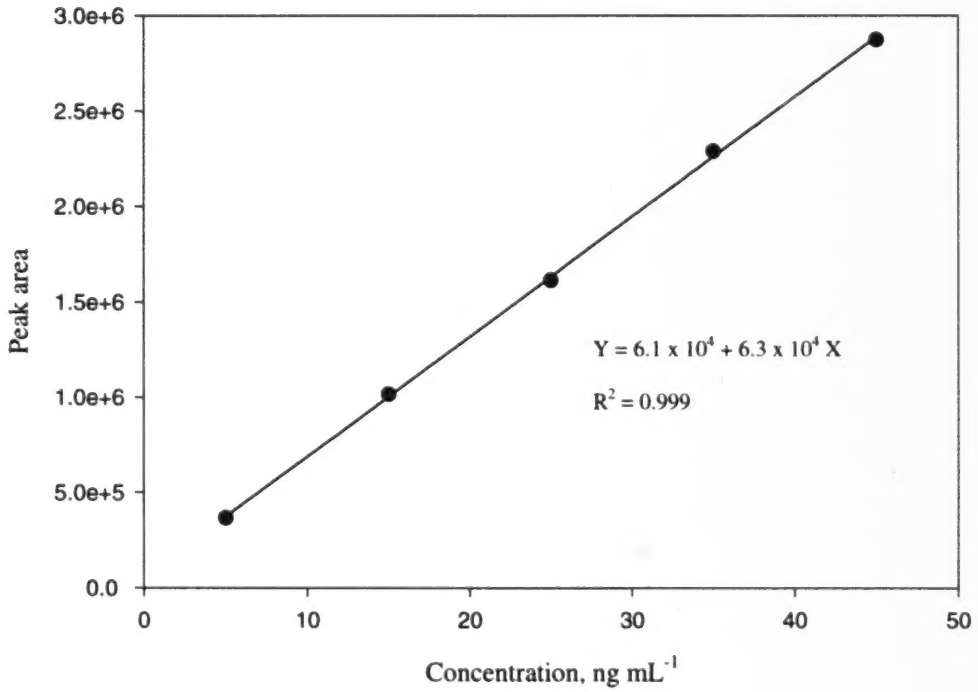

Figure A4: Calibration curve of arsenate standard solutions. Data presented in Table B7 in the Appendix B.

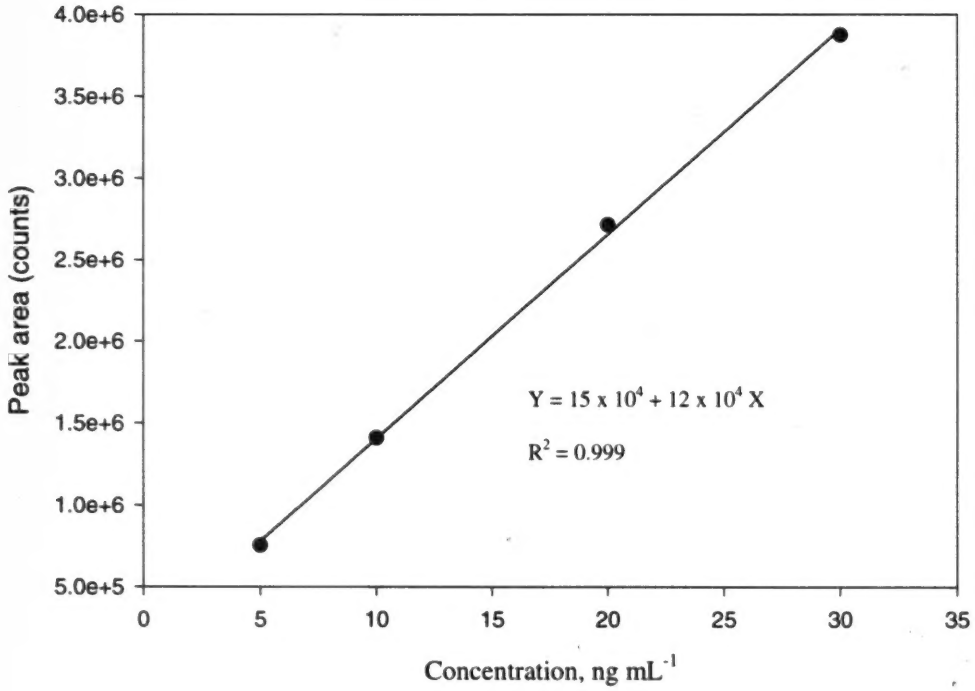

Figure A5: Calibration curve of arsenate standard solutions. Data presented in Table B9 in the Appendix B.

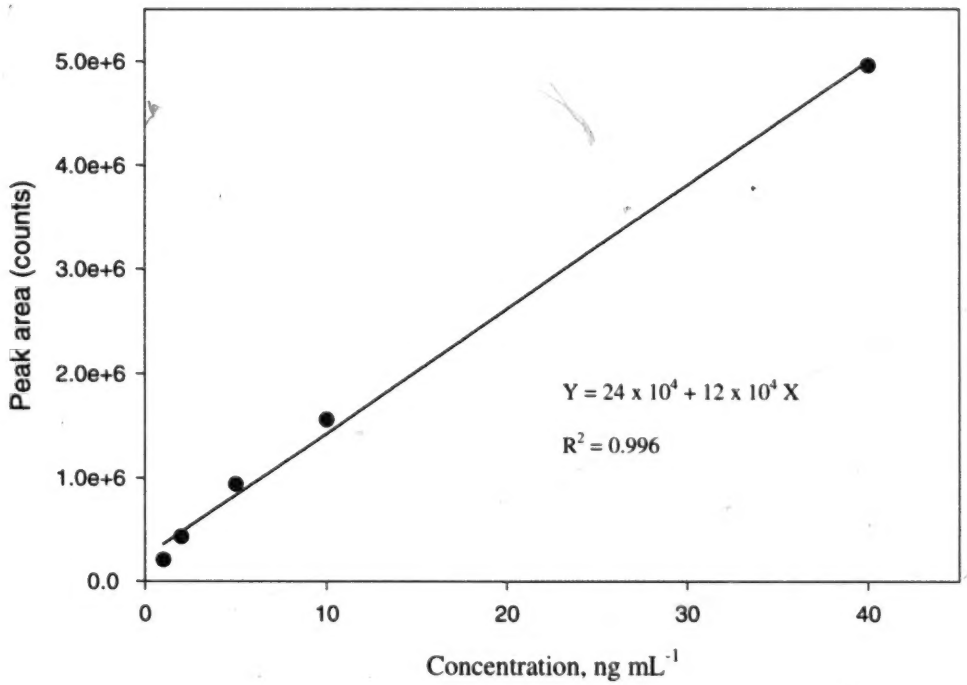

Figure A6: Calibration curve of arsenate standard solutions. Data presented in Table B11 in the Appendix B.

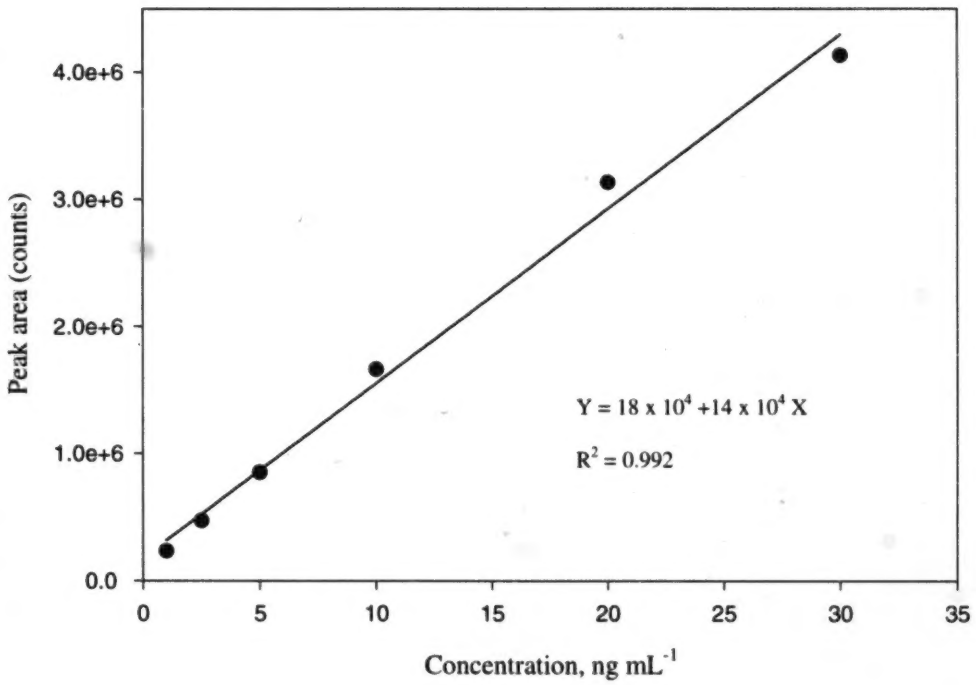

Figure A7: Calibration curve of arsenate standard solutions. Data presented in Table B14 in the Appendix B.

TABLE B1: Calibration curve of arsenate standards: Data for Figure A1

Standards Concentration (ng mL^{-1})	*Peak area (counts)	Standard Deviation	CV(%)
2	188802	2386	1.3
5	433808	37462	8.6
10	801101	23970	3.0
20	1548701	29521	1.9
30	2604269	6113	0.2
40	3465916	139741	4.0

*Average of three injections

TABLE B2: Arsenic concentration in kelp extracts after different digestion treatments. Data for Figure 2.9

Treatment	Concentration Sample 1 (µg/g sample)	Concentration Sample 2 (µg/g sample)	Average	Standard Deviation
Dilution	0.75	0.67	0.71	0.1
Simulated Cooking	0.85	0.64	0.74	0.1
Simulated Gastric	1.2	1.0	1.1	0.1
Basic Digestion	1.6	2.0	1.8	0.2
0.01M TEAH Digestion	1.1	0.53	0.80	0.4
0.1M TEAH Digestion	1.5	2.0	1.8	0.4
Acid Digestion	3.0	1.3	2.2	1.2
Microwave Digestion	14	12	13	1.0

TABLE B3: Calibration curve of arsenate standards: Data for Figure A2

Concentration ($ng\ mL^{-1}$)	*Peak Area (counts)	Standard Deviation	CV(%)
1	108087	4675	4.3
2	195077	2484	1.3
5	577525	1302	0.23
10	1037292	25161	2.4
20	2251606	57687	2.6
30	3461526	50988	1.5

*Average of three injections

TABLE B4: Arsenic concentration in Yakinori extracts after different digestion treatments. Data for Figure 2.10

Treatment	Concentration Sample 1 (µg/g sample)	Concentration Sample 2 (µg/g sample)	Average	Standard Deviation
Dilution	0.93	0.71	0.82	0.2
Simulated Cooking	0.63	0.70	0.67	0.05
Simulated Gastric	0.57	0.76	0.66	0.1
Basic Digestion	0.89	1.3	1.1	0.3
0.01M TEAH Digestion	0.80	0.93	0.87	0.1
0.1M TEAH Digestion	1.1	0.90	0.98	0.1
Acid Digestion	1.2	1.4	1.3	0.2
Microwave Digestion	5.2	5.8	5.5	0.4

TABLE B5: Calibration curve of arsenate standards: Data for Figure A3

Concentration (ng mL^{-1})	*Peak Area (counts)	Standard Deviation	CV(%)
1	91912	6888	7.5
2	184660	16386	8.9
5	418925	18218	4.3
10	814794	29551	3.6
20	1616577	73547	4.5
40	3567627	97957	2.7

*Average of three injections

TABLE B6: Arsenic concentration in kelp extracts after simulated gastrointestinal digestion.

Sample	Non-digested extracts (µg As/g sample)		Enzyme-digested extracts (µg As/g sample)	
	Non MW	MW	Non MW	MW
No. 1	0.53	9.9	0.35	9.6
No. 2	0.48	9.1	0.33	7.9
No. 3	0.40	8.8	0.40	9.0
Average (n=3)	0.47	9.3	0.36	8.8
Standard Deviation	0.1	0.6	0.03	0.9

TABLE B7: Calibration curve of arsenate standards: Data for Figure A4

Concentration (ng mL^{-1})	*Peak Area (counts)	Standard Deviation	CV(%)
5	367606	8872	2.4
15	1015728	10642	1.0
25	1614162	13875	0.9
35	2288855	10784	0.5
45	2873482	21690	0.8

*Average of three injections

TABLE B8: Arsenic concentration in kelp extracts after enzyme-assisted extraction: Data for Figure 3.2

[b]Sample	[a]Extracts		[a]Residues	
	µg As/ g sample	Standard deviation	µg As/ g sample	Standard deviation
[c] CH-1	17	0.2	7.3	0.2
CH-2	18	0.5	5.5	0.1
[d] CP-1	18	0.2	5.6	0.01
CP-2	16	0.3	7.4	0.1
[e] LE-1	16	0.3	5.7	0.2
LE-2	17	0.5	6.2	0.1
[f] CTRL-1	18	0.2	7.5	0.2
CTRL-2	17	0.2	7.3	0.1

[a] Average of three injections

[b] Experimental details are described in section 3.2.4, Chapter 3

[c] CH: Cellulose/Hemicellulase treatment

[d] CP: Cellulose/Pectinase treatment

[e] LE: Lysing enzymes

[f] CTRL: Control samples, non enzymatic treatment

TABLE B9: Calibration curve of arsenate standards: Data for Figure A5

Concentration (ng mL^{-1})	*Peak Area (counts)	Standard Deviation	CV(%)
5	753815	11008	1.5
10	1409651	24898	1.8
20	2714324	55125	2.0
30	3873387	18515	0.5

*Average of three injections

TABLE B10: Arsenic concentration in kelp extracts after enzyme-assisted extraction: Data for Figure 3.4

[b] Sample	[a] Extracts		[a] Residues	
	µg As/ g sample	Standard deviation	µg As/ g sample	Standard deviation
[c] CP-1, 8h	19	0.1	5.9	0.1
CP-2, 8h	15	0.7	5.9	0.1
CP-1, 24h	18	0.2	5.8	0.1
CP-2, 24h	18	0.5	5.5	0.2
[d] CTRL-1, 8h	20	0.8	5.0	0.0
CTRL-2, 8h	20	1.2	4.1	0.1
CTRL-1, 24h	18	1.3	5.5	0.1

[a] Average of three injections

[b] Experimental details are described in section 3.2.4, Chapter 3

[c] CP: Cellulose/Pectinase treatment

[e] CTRL: Control samples, non enzymatic treatment

TABLE B11: Arsenic concentrations in extracts and samples of mussels, tobacco leaves and mushroom after enzyme-assisted extraction. Concentrations in μg arsenic/g sample (n=3).

Sample	Mussels	Tobacco Leaves	Mushroom
As, extracts	1.9 ± 0.3	0.18 ± 0.1	0.38 ± 0.04
As, total digestion	2.1 ± 0.2	0.19 ± 0.03	0.56 ± 0.1
Extraction Efficiency	90%	95%	67%

TABLE B12: Calibration curve of arsenate standards: Data for Figure A6

Concentration (ng mL^{-1})	*Peak Area (counts)	Standard Deviation	CV(%)
1	207002	22624	11
2	429917	23008	5
5	938518	48634	5
10	1564651	21354	1
40	4958285	168982	3

*Average of three injections

TABLE B13: Arsenic concentration in kelp extracts after sequential extraction with bath and probe sonication: Data for Figure 4.4

Sample	[a] µg As/ g sample	Standard deviation
[b]E-1	22	0.1
E-2	19	0.8
E-3	19	0.5
[c]ES-1	1.0	0.2
ES-2	2.3	0.2
ES-3	2.4	0.1
[d]R-1	0.5	0.1
R-2	1.1	0.1
R-3	0.6	0.1

[a] Average of three injections

[b] E1: Arsenic content after extraction of sample 1

[c] ES1: Arsenic content after extraction and sonication of sample 1

[d] R1: Arsenic content in residue of sample 1

TABLE B14: Calibration curve of arsenate standards: Data for Figure A7

Concentration (ng mL^{-1})	Peak Area (counts)	Standard Deviation	CV(%)
1.0	236328	4199	1.8
2.5	475587	3674	0.8
5.0	853369	13108	1.5
10	1663593	65685	3.9
20	3133562	105250	3.4
30	4132454	143182	3.5

*Average of three injections

TABLE B15: Arsenic concentration in kelp extracts after sequential extraction with bath and probe sonication followed by enzyme digestion: Data for Figure 4.5

[b] Sample	[a] µg As/ g sample	Standard deviation
[c]E-1	12	0.2
E-2	11	0.3
E-3	10	0.2
[d]ES-1	5.6	0.5
ES-2	4.8	0.3
ES-3	5.2	0.1
[e]ESI-1	1.4	0.1
ESI-2	1.2	0.2
ESI-3	1.1	0.04
[f]R-1	1.6	0.3
R-2	1.7	0.2
R-3	1.8	0.2

[a] Average of three injections

[b] Experimental details are described in section 4.2.4

[c] E-1: Arsenic content after extraction of sample 1

[d] ES-1: Arsenic content after extraction and sonication of sample 1

[e] ESI-1: Arsenic content after extraction, sonication and enzymatic digestion of sample 1

[f] R-1: Arsenic content in residue of sample 1

TABLE B17: Arsenic concentrations in Nostoc and Yakinori samples after ultrasonic-assisted extraction.

Sample	Yakinori	Noctoc
As, extracts	7.2 ± 1.4	0.3 ± 0.1
As, total digestion	11.3 ± 0.3	2.2 ± 0.2
Extraction Efficiency	68%	14%